

DOUTORAMENTO

MEDICINA

**Estudo de biomarcadores em glioblastomas:
correlação clínica e molecular**

Paulo José de Campos Linhares Vieira

D

2021



Instituições de Acolhimento



Artigo 48º, § 3º

A Faculdade não responde pelas doutrinas expendidas na Dissertação.

(Regulamento da Faculdade de Medicina do Porto – Decreto-Lei nº 19337, de 29 de janeiro de 1931)

Orientador: Professor Doutor Rui Manuel Cardoso Vaz

Professor Catedrático Convidado da Faculdade de Medicina
da Universidade do Porto

Co-orientador: Professor Doutor Bruno Costa

Investigador Auxiliar do Instituto de Investigação em Ciências da Vida e Saúde
(ICVS), Escola de Medicina da Universidade do Minho

JÚRI

Nos termos do disposto do n.º 2 do art.º 18.º do Regulamento dos Terceiros Ciclos de Estudos da Universidade do Porto, a seguir se publicita o júri de doutoramento em Medicina, nomeado pelo despacho de 2021/09/13.

Presidente: Doutora Maria Leonor Martins Soares David

Professora Catedrática da Faculdade de Medicina
da Universidade do Porto

Vogais: Doutor Marcos Daniel de Brito da Silva Barbosa

Professor Auxiliar Convidado da Universidade de Coimbra

Doutora Cláudia Maria Coelho de Faria

Professora Auxiliar Convidada da Universidade de Lisboa

Doutor Nuno Jorge Carvalho Sousa

Professor Catedrático da Universidade do Minho

Doutor Joaquim Luís Ramos Torres Couto Reis

Professor Auxiliar Convidado do Instituto Ciências Biomédicas Abel Salazar
da Universidade do Porto

Doutor Rui Manuel Bento de Almeida Coelho

Professor Catedrático da Faculdade de Medicina
da Universidade do Porto

Doutor Rui Manuel Cardoso Vaz

Professor Catedrático Convidado da Faculdade de Medicina
da Universidade do Porto

Doutor Jorge Filipe Almeida Vieira Lima

Professor Afiliado da Faculdade de Medicina
da Universidade do Porto

CORPO CATEDRÁTICO

Professores Catedráticos Efetivos

Maria Amélia Duarte Ferreira
Patrício Manuel Vieira Araújo Soares Silva
Alberto Manuel Barros da Silva
José Henrique Dias Pinto de Barros
Maria Fátima Machado Henriques Carneiro
Maria Dulce Cordeiro Madeira
Altamiro Manuel Rodrigues Costa Pereira
Manuel Jesus Falcão Pestana Vasconcelos
João Francisco Montenegro Andrade Lima Bernardes
Maria Leonor Martins Soares David
Rui Manuel Lopes Nunes
José Manuel Pereira Dias de Castro Lopes
Joaquim Adelino Correia Ferreira Leite Moreira
Raquel Ângela Silva Soares Lino
Fernando Manuel Mendes Falcão dos Reis
Francisco José Miranda Rodrigues Cruz
José Paulo Alves Vieira de Andrade
Jorge Manuel Silva Junqueira Polónia
José Luís Dias Delgado
Isaura Ferreira Tavares
Fernando Carlos de Landér Schmitt
Acácio Agostinho Gonçalves Rodrigues
Maria de Fátima Moreira Martel
João Tiago de Sousa Pinto Guimarães
José Carlos Lemos machado
José Carlos de Magalhães Silva Cardoso

Professores Catedráticos Jubilados e Aposentados

Alexandre Alberto Guerra Sousa Pinto
Álvaro Jerónimo Leal Machado de Aguiar
António Albino Coelho Marques Abrantes Teixeira

António Augusto Lopes Vaz
António Carlos de Freitas Ribeiro Saraiva
António Carvalho Almeida Coimbra
António Fernandes Oliveira Barbosa Ribeiro Braga
António José Pacheco Palha
António Manuel Sampaio de Araújo Teixeira
Belmiro dos Santos Patrício
Cândido Alves Hipólito Reis
Carlos Rodrigo Magalhães Ramalhão
Cassiano Pena de Abreu e Lima
Deolinda Maria Valente Alves Lima Teixeira
Eduardo Jorge Cunha Rodrigues Pereira
Fernando Távarela Veloso
Francisco Fernando Rocha Gonçalves
Henrique José Ferreira Gonçalves Lecour de Menezes
Isabel Maria Amorim Pereira Ramos
Jorge Manuel Mergulhão Castro Tavares
José Agostinho Marques Lopes
José Carlos Neves da Cunha Areias
José Carvalho de Oliveira
José Eduardo Torres Eckenroth Guimarães
José Fernando Barros Castro Correia
José Luís Medina Vieira
José Manuel Costa Mesquita Guimarães
José Manuel Lopes Teixeira Amarante
Levi Eugénio Ribeiro Guerra
Luís Alberto Martins Gomes de Almeida
Manuel Alberto Coimbra Sobrinho Simões
Manuel António Caldeira Pais Clemente
Manuel Augusto Cardoso de Oliveira
Manuel Machado Rodrigues Gomes
Manuel Maria Paula Barbosa
Maria da Conceição Fernandes Marques Magalhães
Maria Isabel Amorim de Azevedo
Rui Manuel Almeida Mota Cardoso
Rui Manuel Bento de Almeida Coelho
Serafim Correia Pinto Guimarães
Valdemar Miguel Botelho dos Santos Cardoso
Walter Friedrich Alfred Osswald

PUBLICAÇÕES

(Art. 8º do Decreto-lei nº 388/70)

Esta dissertação integra seis trabalhos de primeira autoria: quatro trabalhos originais, um artigo de revisão e uma letter ao editor (cinco publicados e um submetido para publicação).

Artigo 1: Paulo Linhares, Bruno Carvalho, Rita Figueiredo, Rui M Reis, Rui Vaz (2013). *Early pseudoprogession following chemoradiotherapy in glioblastoma patients: the value of RANO evaluation*. Journal of Oncology. doi: 10.1155/2013/690585.

Artigo 2: Paulo Linhares, Marta Viana-Pereira, Mónica Ferreira, Júlia Amorim, Rui Nabiço, Filipe Pinto, Sandra Costa, Rui Vaz, Rui Manuel Reis (2017). *Genetic variants of vascular endothelial growth factor predict risk and survival of gliomas*. Tumor Biology. DOI: 10.1177/1010428318766273.

Artigo 3: Paulo Linhares, Axel Ferreira, Rui Vaz (2019). *The importance of the neutrophil-to-lymphocyte ratio in the prognosis of glioma and its subtypes*. CNS Neuroscience & Therapeutics. doi: 10.1111/cns.13270.

Artigo 4: Paulo Linhares, Mariana Branco, Bruno Carvalho, Patrícia Santos, Bruno M Costa, Rui Vaz (2019). *Serum lactate levels are associated with glioma malignancy grade*. Clinical Neurology and Neurosurgery. doi: 10.1016/j.clineuro.2019.105546.

Artigo 5: Paulo Linhares, Ana Ferreira, Bruno Carvalho, Bruno M Costa, Rui Vaz. *Association Between Red Blood Cell Distribution Width and Survival in Glioblastoma Patients*. Submetido.

Artigo 6: Paulo Linhares, Bruno Carvalho, Rui Vaz, Bruno M Costa (2020). *Glioblastoma: is there any Blood biomarker with true clinical relevance?* International Journal of Molecular Sciences. doi: 10.3390/ijms21165809

Trabalhos publicados em coautoria são igualmente relevantes para diversos tópicos discutidos ao longo deste trabalho e, embora integrem pontos importantes da discussão, são apresentados em Anexo.

Artigo 7: Bruno M Costa, Cláudia Caeiro, Inês Guimarães, Olga Martinho, Teresa Jaraquemada, Isabel Augusto, Lígia Castro, Lígia Osório, Paulo Linhares, Mrinalini Honavar, Mário Resende, Fátima Braga, Ana Silva, Fernando Pardal, Júlia Amorim, Rui Nabiço, Rui Almeida, Carlos Alegria, Manuel Pires, Célia Pinheiro, Ernesto Carvalho, José M Lopes, Paulo Costa, Margarida Damasceno, Rui M Reis (2010). ***Prognostic value of MGMT promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: a portuguese multicentre study***. Oncology Reports. doi: 10.3892/or_00000808

Artigo 8: Bruno M Costa, Marta Viana-Pereira, Ricardo Fernandes, Sandra Costa, Paulo Linhares, Rui Vaz, Céline Pinheiro, Jorge Lima, Paula Soares, Ana Silva, Fernando Pardal, Júlia Amorim, Rui Nabiço, Rui Almeida, Carlos Alegria, Manuel M Pires, Célia Pinheiro, Ernesto Carvalho, Pedro Oliveira, José M Lopes, Rui M Reis (2011). ***Impact of EGFR genetic variants on glioma risk and patient outcome***. Cancer Epidemiology, Biomarkers and Prevention. doi: 10.1158/1055-9965.EPI-11-0340

Artigo 9: Janardan P Pandey, Navtej Kaur, Sandra Costa, Júlia Amorim, Rui Nabiço, Paulo Linhares, Rui Vaz, Marta Viana-Pereira, Rui M Reis (2014). ***Immunoglobulin genes implicated in glioma risk***. Oncoimmunology. doi: 10.4161/onci.28609

Artigo 10: Joana V Castro, Céline Gonçalves, Sandra Costa, Paulo Linhares, Rui Vaz, Ricardo Nabiço, Júlia Amorim, Marta Viana-Pereira, Rui M Reis, Bruno M Costa (2015). ***Impact of TGF- β 1 -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis***. Tumor Biology. doi: 10.1007/s13277-015-3343-0

Artigo 11: Marta Lopes, Bruno Carvalho, Rui Vaz, Paulo Linhares (2017). ***Influence of neutrophil-lymphocyte ratio in prognosis of glioblastoma multiforme***. Journal of Neurooncology. doi: 10.1007/s11060-017-2641-3

- Artigo 12:** Ana Xavier-Magalhães, Ana I Oliveira, Joana V Castro, Marta Pojo, Céline Gonçalves, Tatiana Lourenço, Marta Viana-Pereira, Sandra Costa, Paulo Linhares, Rui Vaz, Rui Nabiço, Júlia Amorim, Afonso A Pinto, Rui M Reis, Bruno M Costa (2017). ***Effects of the functional HOTAIR rs920778 and rs12826786 genetic variants in glioma susceptibility and patient prognosis***. Journal of Neurooncology. doi: 10.1007/s11060-016-2345-0
- Artigo 13:** Marta Viana-Pereira, Daniel A Moreno, Paulo Linhares, Júlia Amorim, Rui Nabiço, Sandra Costa, Rui Vaz, Rui M Reis (2020). ***Replication of GWAS identifies RTEL1, CDKN2A/B, and PHLDB1 SNPs as risk factors in Portuguese glioma patients***. Molecular Biology Reports. doi: 10.1007/s11033-019-05178-8
- Artigo 14:** Pedro V Aguiar, Bruno Carvalho, Rui Vaz, Paulo Linhares (2021). ***Body mass index as an independent prognostic factor in glioblastoma***. Cancer Causes & Control. doi: 10.1007/s10552-020-01388-9

Em cumprimento com o disposto no Decreto-Lei n° 388/70, declara que participou ativamente na recolha e estudo do material incluído e redigiu todos os trabalhos. Esta Dissertação inclui resultados de trabalhos não publicados.

AGRADECIMENTOS

À família, sempre acima de tudo. O grande pilar...

Cláudia, Lia e Pedro,

Gabi,

Meus pais.

Aos amigos, sem exceção, mas lembrando o Túlio, o Paulo R. e o Amândio.

Aos que me permitiram levar este projeto até ao fim. Mestres e amigos.

Muito obrigado Professor Rui Vaz, Professor Rui M Reis e Professor Bruno Costa.

Aos que me ensinaram e com quem continuo a aprender.

Cerejo, Filipe, Josué, Vilarinho e Paulo, o meu muito obrigado, em particular pela amizade.

Aos que ensinei, mas com quem tenho também muito que aprender.

Obrigado a todos, em particular ao Bruno.

Aos meus doentes, que me obrigam todos os dias a pensar.

Às adversidades, que me tornaram mais forte, mais persistente e cada vez com mais vontade de prosseguir.

ÍNDICE

Introdução	31
1. A classificação dos tumores do Sistema Nervoso Central da Organização Mundial de Saúde	33
2. O Glioblastoma	35
3. Os fatores de risco para o desenvolvimento do Glioblastoma	39
4. Os fatores de prognóstico no Glioblastoma	47
5. Os fatores imagiológicos com importância prognóstica	51
6. O tratamento	53
7. Os biomarcadores	55
Objetivos	57
Artigos Publicados	65
1. <i>Early pseudoprogression following chemoradiotherapy in glioblastoma patients: the value of RANO evaluation.</i>	67
2. <i>Genetic variants of vascular endothelial growth factor predict risk and survival of gliomas.</i>	79
3. <i>The importance of the neutrophil-to-lymphocyte ratio in the prognosis of glioma and its subtypes.</i>	93
4. <i>Serum lactate levels are associated with glioma malignancy grade.</i>	97
5. <i>Association between red blood cell distribution width and survival in glioblastoma patients.</i>	103
6. <i>Glioblastoma: Is there any blood biomarker with true clinical relevance?</i>	119
Discussão	137
1. A neuroimagem como biomarcador	139
2. Os modelos moleculares como biomarcadores	147
2.1. Estudos das variantes genéticas do fator de crescimento endotelial vascular	147
2.2. Estudo das variantes de outros genes associados à proliferação vascular e ao crescimento tumoral	152
3. Modelos metabólicos e inflamatórios como biomarcadores	161
Considerações Finais	175
Bibliografia	181
Anexos	189
1. <i>Prognostic value of MGMT promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: A Portuguese multicentre study.</i>	191

2. <i>Impact of EGFR genetic variants on glioma risk and patient outcome.</i>	201
3. <i>Immunoglobulin genes implicated in glioma risk.</i>	213
4. <i>Impact of TGF-β1 -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis.</i>	221
5. <i>Influence of neutrophil-lymphocyte ratio in prognosis of glioblastoma multiforme.</i>	231
6. <i>Effects of the functional HOTAIR rs920778 and rs12826786 genetic variants in glioma susceptibility and patient prognosis.</i>	241
7. <i>Replication of GWAS identifies RTEL1, CDKN2A/B, and PHLDB1 SNPs as risk factors in Portuguese glioma patients.</i>	251
8. <i>Body mass index as an independent prognostic factor in glioblastoma.</i>	263

RESUMO

O glioblastoma é o tumor primário maligno mais frequente, correspondendo a aproximadamente 15% dos tumores intracranianos e a 40-50% dos tumores malignos primários do cérebro. Macroscopicamente é um tumor mal delineado e infiltrativo. A histopatologia é muito variável, sendo tipicamente uma lesão altamente celular. As alterações genéticas típicas do glioblastoma IDH *wildtype* (aproximadamente 90% dos casos de GBM) são as mutações do promotor do gene *TERT*, as deleções homozigóticas do *CDKN2A/CDKN2B*, a perda dos cromossomas 10p e 10q, as alterações do *EGFR*, mutações /deleções do *PTEN* e mutações do *TP53* e *PI3K*. A metilação do promotor do gene *MGMT* ocorre em 30 a 50% dos GBM IDH-*wildtype*, conferindo um prognóstico mais favorável e uma melhor resposta ao tratamento com agentes alquilantes. Os factores mais importantes para o desenvolvimento dos gliomas são a proliferação vascular e a desregulação do ciclo celular. Factores adicionais como a inflamação e a resposta imunológica são também primordiais no desenvolvimento e progressão do glioblastoma. Existem evidências do papel fulcral dos polimorfismos de genes reguladores destes mecanismos na tumorigenese e resposta clínica. Vários factores com importância prognóstica foram descritos para o glioblastoma, entre eles a idade, a extensão da remoção cirúrgica, o estado funcional pré-operatório, a localização, a forma de apresentação e a realização de radioterapia associada a temozolamida concomitante e adjuvante. Factores moleculares como a metilação do gene promotor do *MGMT* e as mutações dos genes *IDH* conferem prognósticos distintos, o mesmo acontecendo com outros factores como rácio neutrófilo-linfócito, o índice RDW dos eritrócitos, os lactatos séricos e o índice de massa corporal, como acabamos por encontrar.

O nosso objetivo foi tentar correlacionar os achados genéticos determinados em sangue periférico com o diagnóstico e evolução clínica dos doentes e identificar biomarcadores de susceptibilidade aumentada para o desenvolvimento de glioblastomas, assim como indicadores de prognóstico e resposta terapêutica.

Começamos por avaliar a pseudoprogressão em ressonância magnética usando dois métodos distintos de avaliação. Obtivemos resultados díspares que foram dependentes do método de avaliação utilizado. Assim concluímos que a pseudoprogressão não representava um fator de prognóstico nos doentes com glioblastoma.

Usando colheitas de sangue periférico dos doentes com glioblastoma fomos estudar a existência de polimorfismos de genes associados à proliferação vascular e ao ciclo celular, avaliando o seu efeito no risco e prognóstico dos doentes com glioblastoma.

O estudo dos polimorfismos do gene *VEGF*, apoiado por estudos adicionais dos genes *EGF/EGFR*, *CDKN2A/B*, *RTEL*, *PHLDB1*, *TERT*, *CCDC26* e *TGF- β 1*, veio demonstrar um risco aumentado de desenvolvimento de gliomas e de pior prognóstico associado a SNPs específicos.

Tendo em conta que os estudos moleculares de base individual são de difícil aplicabilidade clínica, tentámos encontrar associações entre os achados laboratoriais mais comuns, como o rácio neutrófilo-linfócito (RNL), o índice de anisocitose eritrocitária (RDW) e o valor dos lactatos séricos, e o prognóstico dos doentes com glioblastoma. Os nossos resultados mostraram que valores aumentados destes marcadores estavam implicados na diminuição da sobrevivência global, podendo ser usados como biomarcadores de prognóstico.

ABSTRACT

Glioblastoma is the most frequent primary malignant tumor, accounting for approximately 15% of intracranial tumors and 40-50% of primary malignant brain tumors. Macroscopically, it is a poorly delineated and infiltrative tumor. Histopathology is highly variable, typically being a highly cellular lesion. Typical genetic alterations of glioblastoma IDH wild-type (approximately 90% of GBM cases) are *TERT* gene promoter mutations, *CDKN2A/CDKN2B* homozygous deletions, loss of chromosomes 10p and 10q, *EGFR* alterations, mutations/deletions of *PTEN* and mutations of *TP53* and *PI3K*. Methylation of the *MGMT* gene promoter occurs in 30 to 50% of glioblastoma IDH-wildtype, providing a more favorable prognosis and a better response to treatment with alkylating agents. The most important factors for the development of gliomas are vascular proliferation and cell cycle dysregulation. Additional factors such as inflammation and the immune response are also key in the development and progression of glioblastoma. There is evidence of the pivotal role of gene polymorphisms that regulate these mechanisms in tumorigenesis and clinical response. Several factors with prognostic importance have been described for glioblastoma, including age, extent of surgical removal, preoperative functional status, location, presentation form, and radiotherapy associated with concomitant and adjuvant temozolomide. Molecular factors such as methylation of the *MGMT* gene promoter and *IDH* gene mutations provide different prognoses, as do other factors such as neutrophil-lymphocyte ratio, erythrocyte RDW index, serum lactates and body mass index, as we ended up for finding.

Our objective was to try to correlate the genetic findings determined in peripheral blood with the diagnosis and clinical evolution of the patients and to identify biomarkers of increased susceptibility for the development of glioblastomas, as well as indicators of prognosis and therapeutic response.

We begin by evaluating pseudoprogression on magnetic resonance imaging using two distinct evaluation methods. We obtained disparate results that were dependent on the evaluation method used. Thus, we concluded that pseudoprogression was not a prognostic factor in patients with glioblastoma.

Using peripheral blood samples from patients with glioblastoma, we studied the existence of gene polymorphisms associated with vascular proliferation and the cell cycle, evaluating their effect on the risk and prognosis of patients with glioblastoma.

The study of *VEGF* gene polymorphisms, supported by additional studies of the *EGF/EGFR*, *CDKN2A/B*, *RTEL*, *PHLDB1*, *TERT*, *CCDC26* and *TGF- β 1* genes, has shown an increased risk of developing gliomas and worse prognosis associated with SNPs specific.

Considering that individual-based molecular studies are of difficult clinical applicability, we tried to find associations between the most common laboratory findings, such as the neutrophil-lymphocyte ratio (RNL), the erythrocyte anisocytosis index (RDW) and the serum lactate value, and the prognosis of patients with glioblastoma. Our results showed that increased values of these markers were implicated in decreased overall survival and could be used as prognostic biomarkers.

ABREVIATURAS, SIGLAS E ACRÓNIMOS

ADN	Acido Desoxirribonucleico
CAR	<i>Chimeric Antigen Receptor</i>
CDKN	Gene Inibidor da Cinase dependente da Ciclina
EGF	Fator de Crescimento Epidérmico
EGFR	Receptor do Fator de Crescimento Epidérmico
GBM	Glioblastoma
GWAS	<i>Genome Wide Association Studies</i> - Estudos de Associação Alargada do Genoma
Gy	Gray
HER2	Receptor 2 do Fator de Crescimento Epidérmico Humano
HOTAIR	<i>HOX Transcript Antisense RNA</i>
HPFS	<i>Health Professionals Follow-Up Study</i>
IDH	Isocitrato Desidrogenase
IGHG	Imunoglobulina de Cadeia Pesada γ
IMC	Índice de Massa Corporal
LDL	Lipoproteína de Baixa Densidade
MGMT	O-6-Metilguanina-DNA Metiltransferase
MHC	Complexo Principal de Histocompatibilidade
NHS	<i>Nurses' Health Study</i>
NHS II	<i>Nurses' Health Study II</i>
NK	<i>Natural Killer</i>
OMS	Organização Mundial de Saúde
OMS CN5	Classificação da Organização Mundial de Saúde dos Tumores do Sistema Nervoso Central 2021
PD-1	Proteína de Morte Celular Programada 1
PD-L1	Ligando da Proteína de Morte Celular Programada 1
PI3K	Fosfatidilinositol 3-Cinase
PTEN	Fosfatase Homóloga à Tensina
QT	Quimioterapia
RANO	<i>Response Assessment in Neurooncology</i>
RDW	<i>Red Cell Distribution Width</i>
RN	Rácio Neutrófilo-Linfócito

RT Radioterapia
SG Sobrevivência Global
SLP Sobrevivência Livre de Prigressão
SNC Sistema Nervoso Central
SNPs Polimorfismos de Nucleotídeo Único
TERT Transcriptase Reversa da Telomerase
TGFβ Fator de Transformação de Crescimento β
TMZ Temozolamida
TTF Tumor Treating Fields
VEGF Fator de Crescimento Endotelial Vascular
VEGFR Recetor do Fator de Crescimento Endotelial Vascular

PREFÁCIO

O glioblastoma é o tumor cerebral primário maligno mais frequente. Atendendo a algumas das suas características, nomeadamente à agressividade e imprevisibilidade, não foi possível, até ao momento, determinar um método de rastreio ou de diagnóstico precoce. Pelo mesmo motivo se torna difícil prever atempadamente a sua recorrência, situação que, inexoravelmente, irá ocorrer num curto período de tempo.

A pesquisa aturada de alterações que possam indiciar uma fase precoce da gliomagénesse ou da progressão da doença tem recaído em análises complexas e demoradas, mais adequadas ao grupo que ao indivíduo. Embora de importância fulcral no entendimento da doença, esta investigação dilui-se na prática clínica.

Os muitos anos a lidar com esta patologia, com os incessantes fracassos e derrotas, levantaram a questão da necessidade de se procurar contribuir para encontrar algum marcador de fácil acesso e processamento que pudesse numa forma rotineira informar sobre o risco de desenvolvimento, o diagnóstico precoce e o aparecimento da recidiva.

A concretização dum projeto com estas características apenas é plausível num contexto multidisciplinar e pluri-institucional abarcando os diferentes domínios da investigação com recursos a uma investigação básica forte e diferenciada que pudesse beneficiar da experiência acumulada no diagnóstico e tratamento dos doentes e com acesso a um número de casos que permitisse, pelo seu volume, chegar a algumas conclusões.

A oportunidade chegou com o estabelecimento de uma parceria com a Escola de Medicina da Universidade do Minho através do Prof. Doutor Rui Manuel Reis e da sua dedicação aos gliomas cerebrais, em particular ao glioblastoma. O volume de doentes com glioblastoma tratados no Serviço de Neurocirurgia do Centro Hospitalar São João permitia, numa forma multicêntrica que englobava o Centro Hospitalar do Porto, o Hospital de Braga e em menor escala o IPO do Porto, contribuir para a obtenção de um número de doentes que cumpria os desígnios estabelecidos. Assim, passei a integrar uma equipa de investigação pluridisciplinar, que se dividia pelas vertentes básica e clínica, com todos a contribuírem para um maior bem comum, elucidar os diferentes aspetos relacionados com os glioblastomas. A obtenção dos dados clínicos e do material de estudo e a respetiva conservação estava a cargo do

grupo de investigação clínica e o processamento das amostras caberia ao grupo de investigação básica. A preparação dos artigos foi feita de modo conjunto dada a especificidade das partes envolvidas. Desta parceria resultou a elaboração de 12 artigos que, distribuídos pelos diferentes investigadores, culminaram com a realização de várias teses de doutoramento. O Professor Doutor Bruno Costa deu seguimento a este projeto, passando a encabeçar a investigação em gliomas, tendo sido, na segunda fase deste percurso, uma pedra fundamental que levou à sua conclusão.

Numa segunda fase estendeu-se esta parceria ao IPATIMUP, onde a colaboração com o Professor Doutor Jorge Lima e, mais recentemente, com a Professora Doutora Raquel Lima tem permitido a manutenção duma investigação de translação da ciência básica para a ciência clínica, com a participação nalguns projetos desenvolvidos e a desenvolver.

A nossa procura englobou uma avaliação genética, uma avaliação celular e uma avaliação metabólica. Esteve sempre presente a ideia do desenvolvimento de um painel exequível de marcadores, que na sua conjugação ajudassem a responder a um dos maiores desafios relacionados com o glioblastoma: prevê-lo e prever o seu comportamento. A ciência básica tem, a meu ver, um carácter mais populacional, com resultados a médio e longo prazo, que se pretendem generalizar ao grupo. O principal objetivo deste projeto era a pesquisa dum biomarcador de cariz individual, ajustado ao doente em si e não ao grupo em geral.

Este percurso de colaboração com a investigação básica foi acompanhado pelo meu desenvolvimento enquanto médico e neurocirurgião com particular ênfase na neuro-oncologia, onde adquiri competências e desenvolvi áreas como a cirurgia de tumores cerebrais com o doente acordado e a cirurgia de gliomas com fluorescência intra-operatória. Também enquanto clínico fui responsável pelo Grupo Oncológico de Patologia do Sistema Nervoso Central, com reuniões de discussão semanal de todos os doentes orientados nos serviços de Neurocirurgia, Oncologia Médica e Radio-oncologia, além do apoio a outros serviços. Da experiência clínica acumulada resultou a escrita de dois capítulos de livros, um sobre tumores cerebrais em geral e outro sobre glioblastomas. A integração da ciência básica na clínica e a integração dos clínicos na ciência básica é, na minha opinião, fundamental e uma mais valia importante para o desenvolvimento de ambas as áreas.

Este projeto foi a ambição desejada e utópica. Desejada pelo contributo que traria no diagnóstico precoce e na pertinência do tratamento atempado, pré-clínico, que ousasse *apanhar* o tumor na sua fase inicial e confinada, sensível aos tratamentos permitindo sobrevivências alargadas. Utópica na sua dimensão, na vasta procura

e nas constantes respostas negativas. Não perdendo a esperança procuramos respostas nas áreas genética e metabólica. Revimos a literatura. Continuamos com a forte convicção que este é o caminho a percorrer, conjugando cada vez mais a ciência básica com a investigação clínica.

INTRODUÇÃO

A CLASSIFICAÇÃO DOS TUMORES DO SISTEMA NERVOSO CENTRAL DA ORGANIZAÇÃO MUNDIAL DE SAÚDE

Em 1979 a Organização Mundial da Saúde (OMS) publicou a primeira classificação dos tumores do sistema nervoso central (SNC) (Zülch KJ, 1979). Desde essa altura tem vindo a ser incorporado um sistema de graduação que tem como objetivo prever o comportamento biológico do tumor. Durante muito tempo a classificação dos tumores do SNC baseou-se no conceito de que os tumores podiam ser classificados de acordo com as semelhanças microscópicas das suas putativas células de origem e dos seus estados de diferenciação (Louis DN, 2016), baseado no aspeto dos cortes corados com hematoxilina e eosina e na sua expressão imunohistoquímica. Apesar do conhecimento de algumas alterações genéticas, elas não foram incorporadas na classificação da OMS publicada em 2007, tendo apenas sido consideradas na atualização da classificação em 2016, onde os parâmetros moleculares estão já estabelecidos. Não obstante os aspetos histológicos terem alguma acuidade diagnóstica, apresentam uma grande variabilidade inter-observador, não explicam a variabilidade clínica entre entidades morfológicas idênticas e não incorporam aspetos prognósticos, o que criou a necessidade de se identificarem marcadores de diagnóstico mais robustos como os marcadores moleculares. A atualização de 2016 da classificação dos tumores cerebrais da OMS acrescentou parâmetros moleculares definidos, que se adicionaram aos aspetos histológicos já estabelecidos na classificação de 2007, dando um carácter dinâmico, baseado tanto no fenótipo como no genótipo, à classificação, incorporando toda a informação obtida no tecido tumoral, combinando o diagnóstico histológico, o grau histológico e a informação molecular e incluindo também uma gradação traduzindo uma escala de malignidade, com a divisão em 4 graus. Em 2021 foi publicada a quinta edição da classificação dos tumores do SNC da OMS (OMS CNS5), sendo a sexta versão. Foi construída tendo por base a atualização de 2016 da 4.^a edição e o trabalho do *Consortium to Inform Molecular and Practical Approaches to SNC Tumor Taxonomy* (cIMPACT-NOW) (Louis DN, 2021). Introduz essencialmente alterações no diagnóstico molecular, ao mesmo tempo que permanece ligada às outras abordagens estabelecidas para o diagnóstico dos tumores, como a histologia e a imunohistoquímica, enfatizando a importância de um diagnóstico integrado. Mantém, contudo, uma taxonomia híbrida que, segundo os autores, poderá ser uma fase de transição para uma classificação futura mais precisa e, eventualmente, mais homogênea. Na OMS CNS5 algumas famílias tumorais encontram-se agrupadas de acordo com as alterações genéticas que permitem um completo diagnóstico (por exemplo o estado dos genes do grupo isocitrato desidrogenase (IDH) e do grupo

da histona H3); por associações oncogénicas menos fortes como as alterações da via MAPK; por semelhanças histológicas e citogenéticas mesmo com assinaturas moleculares diferentes (por exemplo dentro do grupo de *Outros gliomas, Tumores glioneurais e Tumores neuronais*); ou usando características moleculares para definir novos tipos e subtipos (como no caso do meduloblastoma) (Louis DN, 2021).

Em termos de graduação dos tumores houve também uma mudança para uma graduação dentro do tipo do tumor e não transversal a todos os tipos de tumor, mantendo contudo os 4 graus, tendo esta mudança sido efetuada por 3 motivos:

1. Fornecer maior flexibilidade no uso do grau relativo ao tipo do tumor;
2. Para enfatizar semelhanças biológicas dentro dos tipos de tumor em vez de aproximar o comportamento clínico;
3. Para estar mais em conformidade com as classificações da OMS de tumores não do SNC (Louis DN, 2021).

O GLIOBLASTOMA

O glioblastoma é o tumor cerebral primário maligno mais frequente, correspondendo a aproximadamente 15% dos tumores intracranianos e a 40-50% dos tumores malignos primários do cérebro (Louis DN, 2016). Na maioria dos países europeus e na América do Norte tem uma incidência na ordem dos 3-4 novos casos por 100 000 habitantes por ano (Louis DN, 2016). Apresenta um pico de incidência entre os 55 e os 85 anos de idade, um predomínio no sexo masculino (Louis DN, 2016; Kaye A, 2001; Bernstein M, 2000) e uma idade média ao diagnóstico de 65 anos (Tan AC, 2020). A incidência no género masculino é de 1,6 vezes maior que no género feminino, e 2,0 vezes maior nos caucasianos em comparação com africanos e afro-americanos, tendo menor incidência em asiáticos e índios americanos (Juweid M et al, 2017).

Kang H e colaboradores (col.) determinaram as características epidemiológicas e a sobrevivência de doentes com tumores cerebrais primários e outros tumores do SNC na Coreia e compararam os resultados com os dos Estados Unidos. Verificaram que a taxa de incidência padronizada para a idade dos tumores malignos foi significativamente menor (4,27/100 000) do que nos Estados Unidos (7,08/100 000) (Kang H et al, 2020). Globalmente, a incidência do glioblastoma é mais alta na América do Norte, Austrália e Europa do Norte e Ocidental (Tan AC, 2020). A taxa de incidência relativamente mais baixa de tumores primários malignos do SNC foi observada não apenas nos países do leste asiático, mas também em países da Ásia Ocidental. Embora a causa aparente não seja clara, vários relatos sugerem que disparidades na etnia, estilo de vida, ambiente, fatores hereditários e composição da população possam ser responsáveis por estas variações (Kang H et al, 2020), não se devendo, por consequência, a uma menor taxa de diagnósticos efetuados.

Como outras doenças complexas, acredita-se que o glioma resulte da combinação de fatores genéticos e ambientais (Pandey JP, 2014).

Historicamente, a origem glial dos glioblastomas foi reconhecida pela primeira vez por Virchow em 1863 e o termo “glioblastoma multiforme” foi atribuído por Mallory em 1914. O seu desenvolvimento surge a partir de células progenitoras neurogliais (Tan AC, 2020).

Macroscopicamente o glioblastoma é mal delineado sendo a superfície de corte variável na cor, muitas vezes com a massa tumoral periférica acinzentada e com áreas centrais de necrose amarelada. A zona hipercelular periférica surge como uma

borda suave cinzenta a rosa ou uma faixa cinzenta de tecido tumoral. Normalmente há um pontuado avermelhado e acastanhado traduzindo focos de hemorragia recente e antiga (Louis DN, 2016).

A histopatologia é muito variável, o que levou à denominação de *glioblastoma multiforme*. Tipicamente é uma lesão altamente celular, geralmente composta por células tumorais pouco diferenciadas, pleomórficas, com atipia nuclear e atividade mitótica ativa, sendo a proliferação microvascular proeminente e a necrose elementos essenciais para o diagnóstico, que é frequentemente baseado na identificação de um padrão tecidual e não apenas pela existência de tipos celulares específicos (Louis DN, 2016).

Também importante para a caracterização diagnóstica do glioblastoma é a determinação do estado da mutação do gene *IDH*, resultando em dois subgrupos distintos, a saber, glioblastoma *IDH-wildtype* e glioblastoma *IDH-mutante* (Tan AC, 2020). Apesar da OMS CNS5 ter abolido a entidade glioblastoma *IDH-mutante*, passando estes tumores a serem classificados como astrocitomas *IDH-mutante* grau 4 (Louis DN, 2021), consideramos nesta dissertação a atualização de 2016 da classificação dos tumores do SNC para fins de descrição e discussão. As alterações genéticas típicas do glioblastoma *IDH-wildtype* são as mutações do promotor do gene da transcriptase reversa da telomerase (*TERT*), as deleções homozigóticas do gene inibidor da cinase dependente da ciclina (*CDKN*) *CDKN2A/CDKN2B*, a perda dos cromossomos 10p e 10q, as alterações do recetor do fator de crescimento epidérmico (*EGFR*) (mutações, rearranjos e /ou amplificações), mutações/deleções do gene da fosfatase homóloga à tensina (*PTEN*) e mutações do gene *TP53* e do gene fosfatidilinositol 3-cinase (*PI3K*) (Louis DN, 2016). Com base na mutação do *IDH*, o *wildtype* corresponde ao glioblastoma primário, sem tumor precursor identificável, compreendendo 90% dos glioblastomas (Louis DN, 2016). A forma mutante que corresponde aos restantes 10% classifica o glioblastoma em secundário, tendo origem num astrocitoma difuso ou anaplásico precursor (Tan AC, 2020). A metilação do gene promotor do O6-metilguanina-DNA metiltransferase (*MGMT*) ocorre em 30 a 50% dos glioblastomas *IDH-wildtype*, conferindo uma melhor resposta ao tratamento com agentes alquilantes e um prognóstico mais favorável. Recentemente, a classificação da OMS CNS5 acabou por retirar da classificação o glioblastoma *IDH-mutante*, optando por englobá-lo no tipo astrocitoma *IDH-mutante*. Considerou que o glioblastoma *IDH wildtype* deve ser diagnosticado no contexto de um glioma astrocitário, difuso, *IDH wildtype* se houver microproliferação vascular ou necrose ou mutação do promotor do gene *TERT* ou amplificação do gene *EGFR* ou alteração do número de cópias do cromossoma +7/-10 (Louis DN, 2021).

Clinicamente o glioblastoma manifesta-se essencialmente por quatro quadros distintos: hipertensão intracraniana, défices neurológicos focais, epilepsia e sintomas pouco específicos como fadiga ou alterações cognitivas e comportamentais. Muitos doentes têm mais que um sintoma simultaneamente e normalmente apresentam mais sintomas do que os que relatam ou são detetados pelo médico. Dois ou mais sintomas relacionados entre si e que ocorrem juntos podem ser referidos como um agrupamento de sintomas, sendo que as associações entre os sintomas dentro de um grupo de sintomas são mais fortes do que as associações entre diferentes grupos de sintomas e/ou sintomas separados (Coomans MB, 2019). Estes grupos de sintomas têm uma relação direta com a qualidade de vida. Coomans e col. identificaram quatro grupos de sintomas, um grupo de dor, um grupo motor, um grupo de fadiga e um grupo gastrointestinal/convulsivo/bexiga. O grupo de fadiga, que engloba o cansaço e a sonolência foi o mais prevalente, seguido do grupo motor (Coomans MB, 2019).

Estão descritos alguns fatores de risco e de prognóstico no glioblastoma, sendo, no momento, os mais relevantes a metilação do gene promotor do *MGMT* e as mutações do gene *IDH* no prognóstico.

OS FATORES DE RISCO PARA O DESENVOLVIMENTO DO GLIOBLASTOMA

A etiologia do glioblastoma é desconhecida (Nelson JS, 2012) e há poucos fatores de risco conhecidos para o seu desenvolvimento. Exclui-se aqui uma pequena quantidade associada a síndromes genéticas hereditárias como a síndrome de Turcot, a síndrome de Li-Fraumeni, a neurofibromatose e a síndrome de Lynch que representam apenas cerca de 1% dos casos.

Dos fatores de risco reportados salientam-se a radiação ionizante, a diminuição da susceptibilidade às alergias, fatores e genes imunológicos e polimorfismos de nucleotídeo único detetados por estudos de associação genômica alargada (GWAS) (Thakkar JP, 2014). Rogers LR e col. encontraram a síndrome metabólica numa frequência ligeiramente maior em doentes com diagnóstico de glioblastoma quando comparado com a população em geral (Rogers LR, 2020).

Nelson JS num estudo epidemiológico prospetivo de longo prazo em homens nipo-americanos, embora com um número reduzido de doentes, encontrou o consumo alimentar de açúcar e o trabalho habitual com exposição média ou alta ao tetracloreto de carbono como fatores independentes associados ao desenvolvimento de glioblastomas (Nelson JS, 2012).

Utilizando uma randomização Mendeliana, Howell AE e col. avaliaram se as associações de 36 fatores de risco de glioma mostravam evidências de uma relação causal (Howell AE, 2020). Obtiveram um resultado positivo entre o comprimento dos telómeros de leucócitos, doença alérgica, consumo de álcool, obesidade extrema na infância, níveis de lipoproteína de baixa densidade (LDL) e triglicerídeos e gliomas.

Cote e col analisaram a relação de ingestão de chá e café com risco de glioma em três grandes estudos de coorte prospetivos, o *Nurses' Health Study* (NHS), *Nurses' Health Study II* (NHSII) e *Health Professionals Follow-Up Study* (HPFS) e concluíram que a ingestão de chá tem uma associação inversa marginal com o risco de glioma e a ingestão de café com cafeína ou descafeinado e o consumo total de café não tiveram impacto no risco (Cote DJ 2, 2019).

A exposição à radiação ionizante é o fator de risco mais fortemente associado ao glioblastoma e é o único fator de risco potencialmente modificável conhecido (Tan AC, 2020). A radiação ionizante induz vários tipos de danos no ácido desoxirribonucleico

(ADN), particularmente quebras da cadeia dupla, que são as principais ameaças à integridade genómica das células. Uma associação entre a radiação ionizante de alta dose e o desenvolvimento de tumores cerebrais foi observada em estudos de sobreviventes de bomba atómica, dados de testes nucleares radioativos, radiação terapêutica para situações malignas e benignas e estudos ocupacionais e ambientais (Prabhakaran N, 2019). Foi demonstrado que a radiação ionizante promove a migração e invasão de linhas celulares de glioblastoma humano *in vitro*, sugerindo que este efeito pode permitir que algumas células cancerosas se afastem da área irradiada, reduzindo assim sua exposição à radiação nas subseqüentes sessões de irradiação e, em geral, à eficácia do tratamento (Gauthier LR, 2020).

A suscetibilidade genética de glioblastomas vem de síndromes genéticas raras, história familiar de múltiplos cânceres e alterações cromossômicas. Os vários genes associados ao ciclo celular, à reparação celular e ao metabolismo apresentam resultados muitas vezes discrepantes quanto ao risco de gliomagenese.

Os polimorfismos de genes são um factor que pode aumentar a susceptibilidade ao desenvolvimento do glioblastoma. Os polimorfismos de nucleotídeo único (SNPs) não é comum mudarem durante o processo de carcinogénese (Gu J, 2009) e apresentam uma baixa taxa de mutação, o que os torna biomarcadores adequados na avaliação de risco, sendo importante perceber o seu papel em vias específicas como por exemplo da reparação do ADN, ciclo celular, inflamação, angiogénese e metabolismo.

Os genes associados ao ciclo celular têm mostrado respostas variáveis no risco do desenvolvimento de glioblastomas. A via do *EGFR* desempenha funções importantes na regulação do crescimento celular, apoptose e diferenciação, um processo que é rigidamente regulado em células epiteliais normais. A sinalização aberrante do *EGFR* pode, em última análise, afetar as principais características do cancro, incluindo o crescimento tumoral, a invasão, a malignidade e o prognóstico. Além dessas alterações moleculares somáticas, variantes polimórficas germinativas na via *EGF/EGFR* também foram implicadas no risco de tumor, resposta ao tratamento e resultado do doente. O polimorfismo *EGF* (*EGF p61*) está associado com risco de glioma, o que aumenta a hipótese crescente que polimorfismos na via do *EGFR* podem ser relevantes no contexto dos gliomas (Costa BM, 2011). Foi observada uma associação positiva com o *EGF/EGFR* em muitos dos estudos publicados, como é o caso do *EGF+61A/G* em que a variante G se associa a aumento do risco de glioma, o mesmo não acontecendo com os genes *PTEN* e *TP53* que mostraram resultados contraditórios (Gu J, 2009). Costa e col. em 2007 num estudo que envolveu 190 doentes com glioma e 570 controlos mostraram o papel do polimorfismo *EGF + 61* como um fator de suscetibilidade para o desenvolvimento de glioblastomas (Costa

B, 2007). Já em 2010, Bao e col. tinham mostrado uma associação entre o genótipo *EGF +61 AA* e o risco aumentado de glioblastoma na população asiática, uma vez que encontraram este genótipo numa frequência significativamente superior nos doentes com este tipo de tumor do que na população geral e do que na população com outro tipo de tumores gliais (Bao G, 2010). Subsequentemente, o mesmo grupo estudou o polimorfismo do gene *VEGF +936* tendo verificado que os doentes com glioblastoma tiveram uma frequência significativamente maior do genótipo TT do que os controlos e do que os doentes com outros gliomas (Bao G, 2011). Os polimorfismos do *EGFR* foram também associados ao risco de desenvolvimento de glioblastoma. Trabalhos do nosso grupo implicaram o *EGFR -191C/A* e os polimorfismos de repetição $(CA)_n$ do intrão 1 do *EGFR* como fatores de risco para gliomas (Costa B, 2011). Uma meta-análise efetuada por Chen e col. em 2014 mostrou que o polimorfismo *EGF + 61G/A* está associado ao risco de desenvolvimento de glioblastoma (Chen X, 2014).

O fator de crescimento endotelial vascular (VEGF) é o principal fator angiogénico tanto no desenvolvimento embrionário como no crescimento tumoral. Está sobre regulado no glioblastoma e seus recetores estão sobreexpressos nos vasos tumorais (Linhares P, 2018). Estudos recentes têm mostrado que alguns polimorfismos de nucleotídeo único (SNPs) de genes relacionados com o *VEGF/VEGFR* podem contribuir para a modulação dos níveis séricos de VEGF, possivelmente influenciando o risco e o prognóstico de tumores sólidos, incluindo glioma (Linhares P, 2018). Num estudo desenvolvido em 2018 apontamos que diferentes SNPs do *VEGF* aumentam muito o risco de desenvolver gliomas, e especificamente glioblastoma, particularmente a presença da variante rs833061 (Linhares P, 2018) sendo um dos estudos desenvolvidos no âmbito desta dissertação.

Dos genes associados à reparação de danos no DNA, o *ERCC1*, *ERCC2* e *MGMT*, foram consistentemente associados com risco de glioma (Gu J, 2009). O gene *ERCC1* codifica uma proteína que atua na via de reparação do ADN por excisão de nucleotídeos em lesões como as induzidas por luz ultravioleta ou por agentes como a cisplatina. O gene *ERCC2* codifica a proteína XPD que faz parte do complexo geral do factor de transcrição (TFIIH) que está envolvido na transcrição de genes e na reparação do ADN danificado. O gene *MGMT* localiza-se no cromossoma 10q26 e codifica uma proteína reparadora do ADN que remove grupos alquil da posição O6 da guanina, um importante local de alquilação do ADN (Hegi ME, 2005). Rodriguez-Hernandez e col. mostraram que os polimorfismos *MLH1 rs1800734* e *ERCC2 rs13181* podem constituir fatores de susceptibilidade ao glioblastoma. Os portadores do genótipo *ERCC2 Gln/Gln* foram associados a um risco inferior, enquanto os portadores do genótipo *MLH1 AA* foram associados com um risco aumentado de glioblastoma. Além disso, o haplótipo contendo o alelo C do polimorfismo *ERCC2*

rs13181 e o alelo T do polimorfismo *ERCC1 rs11615* foi significativamente associado a um efeito protetor de desenvolver glioblastoma (Rodriguez-Hernandez, 2013).

A associação dos genes associados ao metabolismo com o risco de glioma é ainda mais inconsistente, havendo apenas alguma evidência duma associação positiva com os genes *SOD2*, *SOD3* e *CAT* (Gu J, 2009).

Em 2019, dando continuidade ao trabalho desenvolvido com os polimorfismos específicos de nucleotídeo único, Viana-Pereira e col. mostraram que os SNPs *rs4977756 (CDKN2A/B)*, *rs6010620 (RTEL1)*, *rs498872 (PHLDB1)*, *rs2736100 (TERT)* e *rs4295627 (CCDC26)* estavam associados à suscetibilidade ao glioma sendo potenciais biomarcadores de risco. O genótipo GA do *rs6010620 (RTEL1)* foi associado a uma diminuição do risco de glioblastoma e o genótipo GA do *rs498872 (PHLDB1)* foi associado a um risco aumentado de glioblastoma (Viana-Pereira M, 2019).

Já em 2016 Ghasimi e col. tinham mostrado que os polimorfismos *rs17172430 (EGFR)* e *rs1412829 (CDKN2B)* mostraram uma tendência de associação com o aumento do número de cópias do EGFR, e que os polimorfismos *rs4977756 (CDKN2A/B)* e *rs1412829 (CDKN2A/B)* apresentavam uma associação inversa com a expressão da forma mutada do IDH1 (Ghasimi S, 2016).

Os resultados dum estudo caso-controlo na população chinesa por Han e col. mostraram que os genótipos CC do polimorfismo *rs2297440 (RTEL1)* e GG do polimorfismo *rs6010620 (RTEL1)* aumentavam significativamente o risco de glioblastoma (Yang B, 2015). Já o genótipo TT do *rs17748 (TREH)* e o genótipo TT do *rs498872 (PHLDB1)* associavam-se com um risco diminuído de GBM no modelo recessivo. Também verificaram que o alelo C variante *TREH rs17748* apresentou um risco aumentado em homens no modelo dominante (Yang B, 2015). Na mesma população, Zhang e col avaliaram se os polimorfismos de nucleotídeo único (SNPs) do gene *EFEMP1*, que tem ação anti-angiogénica, estavam associados à suscetibilidade ao glioma (Zhang S, 2015). Encontraram quatro SNPs associados ao risco de desenvolvimento de glioblastomas, *rs1346787*, *rs3791679*, *rs1346786* e *rs3791675*. Na análise de haplótipos, o haplótipo AA e o haplótipo GG mostraram uma associação significativa com o glioma havendo um aumento de 44% e 65% do risco de glioma em comparação com os não portadores.

Também os polimorfismos do gene *TERT*, particularmente o *rs2736100*, foram associados a susceptibilidade aumentada de desenvolvimento de glioblastoma (Zhou P, 2014).

Outro grupo de genes que tem sido associado ao risco de desenvolvimento de glioblastoma são os relacionados com o sistema imune. Num estudo publicado em 2014 com a participação do nosso grupo, Pandey e col. mostraram que os doentes portadores do alelo GM 3 da IgG1 têm risco 3 vezes maior de glioma de alto grau do que os não portadores (Pandey JP, 2014). Os alelos GM interagem epistaticamente com alelos FcγRIIIa expressos em células *natural killer* e medeiam a citotoxicidade celular dependente de anticorpos de células cancerosas com sobreexpressão de EGFR e HER2 (Namboodiri AM, 2011).

Saunders e col usaram um estudo de associação do fenómeno (PheWAS) de randomização mendeliana (MR) e analisaram 316 fenótipos, representados por 8387 variantes genéticas e dados genéticos resumidos de um GWAS de 12.488 casos de glioma e 18.169 controlos. Associações sugestivas foram observadas entre o comprimento dos telómeros de leucócitos com os glioblastomas, mas a associação foi impulsionada principalmente pela variante *TERT* rs2736100. O colesterol sérico LDL e a HbA1C plasmática mostraram associações sugestivas com glioma, ambas as associações sendo dependentes de variantes genéticas únicas (Saunders CN, 2020). Não encontraram outros fatores de risco para o desenvolvimento de glioblastomas.

Estudos de GWAS mostraram que variantes herdadas em algumas regiões cromossómicas, como 20q13.3, 5p15.3 e 11q23.3, têm uma associação significativa com o risco de glioma (Yang B, 2015).

Thakkar e col não encontraram evidências substanciais de associação de glioblastoma com características do estilo de vida, como o tabagismo, o consumo de álcool, o uso de drogas de qualquer tipo ou a exposição alimentar a compostos N-nitroso (Thakkar JP, 2014). Cote DJ e col. encontraram uma relação inversa entre a hiperlipidemia e o risco de glioma (Cote DJ 1, 2019). Montemurro N e col. efetuaram uma revisão sistemática tendo encontrado 3 artigos que forneciam evidência sobre a diabetes *mellitus* tipo 2 e a hiperglicemia e o risco de desenvolvimento de glioblastomas. Dois dos artigos não encontraram qualquer relação (Barami K, 2017; Disney-Hogg L, 2018) enquanto o terceiro (Seliger C, 2016) mostrou uma diminuição do risco de desenvolvimento de glioblastoma (Montemuro N, 2020). Schwartzbaum e col. usando os dados das coortes *Apolipoprotein MOrtality RiSk* (AMORIS) e do *Metabolic syndrome and Cancer project* (Me-Can) concluíram que os níveis de glicose no sangue pré-diagnóstico foram inversamente relacionados com o risco de glioma (Schwartzbaum J, 2017).

O sistema imunológico desempenha um papel vital na formação e estabelecimento de tumores, tendo funções de proteção do hospedeiro e de iniciação tumoral (Tivnan A, 2017). O sistema nervoso central está sujeito a uma imunovigilância ativa e a uma

vigorosa resposta imunológica (Lim M, 2018). Há uma primeira fase de eliminação em que o sistema imune elimina as células que se transformam, uma segunda fase de equilíbrio em que a imunomodulação ocorre por meio da mutação de antígenos associados às células, regulação negativa, deleção e/ou sobrevivência seletiva de determinadas subpopulações de antígenos negativas ou positivas, envolvendo regulação negativa do complexo principal de histocompatibilidade (MHC) - classe II, expressão aumentada da proteína 4 associada a linfócitos T citotóxicos (CTLA-4), da proteína de morte celular programada (PD-1), IL-10 e TGF- β , além do recrutamento de células T reguladoras para amortecer a resposta imunológica e uma terceira fase de escape quando os tumores editados imunologicamente crescem e se apresentam num ambiente clínico, estabelecendo um microambiente tumoral imunossupressor onde a actividade tumor infiltrante dos linfócitos é suprimida (Tivnan A, 2017). Bloch e col. mostraram que macrófagos isolados do sangue periférico de doentes com glioblastoma expressavam níveis aumentados de PD-L1, um ligando que ativa o receptor ponto de verificação imunológica da proteína de morte celular programada 1 (PD-1) que restringe a atividade das células T citotóxicas (Bloch O, 2013). Também o STAT3 parece ter um papel de sinalização na supressão da actividade das células imunológicas nos doentes com glioblastoma. A expressão STAT3 dos espécimes de glioblastoma humano parece ser provocada pela IL-10 nas células tumorais e nas células imunológicas. De facto, fatores solúveis, nomeadamente o fator de transformação de crescimento β (TGF β), a IL-10 e as prostaglandinas, foram os primeiros mediadores imunossupressores identificados em doentes com glioblastoma, embora estudos subsequentes também tenham indicado que as interações diretas entre as células de glioblastoma e as células do sistema imunológico estão envolvidas nos mecanismos de escape imunológico, com supressão das células NK (*natural killer*). Estas interações resultam na supressão da atividade das células *natural killer* (NK), mediada por moléculas HLA atípicas (incluindo HLA- E51 e HLA- G52), indução direta de apoptose das células do sistema imunológico via interações da superfamília de recetores do fator de necrose tumoral membro 6 (TNFRSF6, comumente conhecido como FAS) – FAS ligando (FASL) ou atingimento dos pontos de verificação de células T pelos ligandos PD-1 (Lim M, 2018).

A susceptibilidade às alergias e a associação inversa com o glioblastoma também tem sido documentado, embora não sejam cabalmente conhecidos os motivos biológicos subjacentes (Tan AC, 2020). Alguns estudos epidemiológicos mostraram que adultos com glioma têm 1,5 a 4 vezes menos probabilidade do que os controlos de relatar uma variedade de alergias, o que transforma a falta de alergias num dos fatores de risco mais consistentes reportado para o desenvolvimento de gliomas (Wiemels JL, 2009).

Mantém-se inconsistente a relação do uso de telemóveis com o desenvolvimento do glioblastoma (Thakkar JP, 2014). O maior estudo multicêntrico de caso-controlo de base populacional, o estudo *Interphone*, realizado em 13 países, apoiou a ideia de que o uso de telefones móveis não tem risco aumentado de glioma, contudo conclui que é necessária mais investigação sobre os possíveis efeitos do uso maciço prolongado dos telemóveis no desenvolvimento dos tumores cerebrais (Kalakoti P, 2016). Num estudo de base populacional australiano, Karipidis K e col examinaram as tendências dos tumores cerebrais nos períodos 1982-1992, 1993-2002 e 2003-2013 e compararam a incidência observada durante o período de uso substancial do telefone móvel (2003-2013) com a incidência prevista para o mesmo período, aplicando vários riscos relativos, períodos de latência e cenários de uso do telefone móvel. Não encontraram aumento em nenhum tipo histológico de tumor cerebral ou localização de glioma que pudesse ser atribuído à utilização dos telemóveis (Karipidis K, 2018).

O nível socioeconómico tem sido associado a vários tipos de cancro. Porter e col numa análise dos dados do Programa SEER (*Surveillance, Epidemiology and End Results*) verificaram que o género masculino, a idade avançada, a raça branca e um maior nível socioeconómico foram positivamente correlacionados com o risco de glioblastoma (Porter AB, 2015).

As únicas associações de fatores de risco validadas foram o aumento do risco após a radiação ionizante para a cabeça e pescoço e uma diminuição do risco entre indivíduos com histórico de alergias e/ou doença atópica (Louis DN, 2016). Todas as outras investigações efetuadas, incluindo a influência potencial da radiação não ionizante (por exemplo, o uso de telemóveis) e as exposições laborais, foram inconclusivas ou com resultados negativos.

OS FATORES DE PROGNÓSTICO NO GLIOBLASTOMA

Vários fatores com importância prognóstica foram descritos para o glioblastoma, entre eles a idade, a extensão da remoção cirúrgica, o estado funcional pré-operatório, a localização, a forma de apresentação e a realização de radioterapia associada a temozolamida concomitante e adjuvante (Dobran M, 2018). Fatores moleculares como a metilação do gene promotor do *MGMT* e as mutações dos genes *IDH* conferem prognósticos distintos. Outros fatores, com diferentes graus de evidência, também têm sido descritos, como por exemplo, o rácio neutrófilo-linfócito (Bambury RM, 2013; Han S, 2015; Weng W, 2018), os lactatos séricos (Shih CC, 2017; Ioannoni E, 2020) e o índice de massa corporal (Cote DJ, 2018; Potharaju M, 2018; Jones LW, 2010). Recentemente, Schuss e col adicionaram a ventilação mecânica prolongada pós-operatória (>24 horas) como fator de risco independente de pior sobrevivência global (Schuss P, 2020). Conjugando alguns valores hematológicos, Xie e col. estabeleceram um *score* inflamatório sistémico com importância prognóstica na sobrevivência dos doentes com glioblastoma (Xie T, 2020).

O silenciamento epigenético do gene *MGMT* pela metilação do promotor está associado à perda de expressão do *MGMT* e diminuição da atividade de reparação do ADN (Hegi ME, 2005). A proteína *MGMT* reverte rapidamente a alquilação na posição O6 da guanina, evitando assim a formação de ligações cruzadas letais (Esteller M, 2000).

Este silenciamento tem sido reconhecido como um factor importante preditor da resposta terapêutica em doentes com glioblastoma tratados com agentes alquilantes (Hau P, 2007) além da radioterapia, conferindo um aumento da sobrevivência global (Hegi ME, 2005).

As mutações nos genes *IDH1* ou *IDH2* têm sido frequentemente identificadas em gliomas. As mutações do *IDH1* e do *IDH2* são restritas ao exão 4, no codão 132 para *IDH1* e codão 172 para *IDH2*. A enzima mutante resultante ganha uma nova capacidade catalítica para produzir 2-hidroxioglutarato. Foi demonstrado que estas mutações se correlacionam com um resultado de sobrevivência mais favorável (Amelot A, 2015). Parsons e col. relataram que a mutação *IDH1* foi associada com uma sobrevivência global significativamente mais longa em doentes com GBM recém-diagnosticado (Parsons DW, 2008). No estudo fase II BELOB a análise univariada mostrou que o estado de mutação *IDH1* foi um fator que se correlacionou significativamente com a sobrevivência global, contudo a análise multivariada não

revelou impacto estatisticamente significativo da mutação IDH1 na sobrevivência global (Estudo BELOB fase II).

Vários SNPs do *VEGF* parecem ser marcadores de prognóstico e de sobrevivência em doentes com glioblastoma, tendo Djan e col. mostrado que o polimorfismo do gene + 405C> G *VEGF* pode ser usado como marcador genético de prognóstico de sobrevivência global em doentes com glioblastoma (Djan I, 2019). Resultando de uma das nossas linhas de investigação, parece que, em particular, os SNPs rs3024994 heterozigotos e os rs1005230 homozigotos conferem um pior prognóstico para estes doentes (Linhares P, 2018).

O microambiente imunológico é composto por células imunológicas associadas ao glioma como a microglia, os macrófagos e as células B e fatores imunorreguladores como a IL-6, a IL-10 e o TGF β , que regulam a progressão do glioma (Wang J-J, 2021). Wang e col. desenvolveram um modelo de prognóstico baseado nos genes relacionados com o sistema imunológico. Definiram um conjunto de 5 genes (*CDK4*, *IL24*, *NOX4*, *BMP2* e *BIRC5*) que apresentavam níveis mais elevados no tecido tumoral que no tecido normal.

O TGF- β , uma citocina multifuncional, está envolvida na regulação de vários processos imunomoduladores que desempenham uma função fundamental em vários processos celulares, como proliferação, diferenciação, apoptose, angiogénese, progressão tumoral e produção de matriz extracelular (Massague J, 2008; Birch JL, 2020; Burghardt I, 2021). A desregulação da sinalização de TGF- β foi implicada no cancro, onde foi demonstrado que o TGF- β tem uma dupla função. Pode atuar como um forte inibidor da proliferação de astrócitos e células epiteliais, sendo considerado um fator supressor tumoral, mas em alguns tipos de tumor, como no glioma de alto grau, o TGF- β atua como um fator oncogénico contribuindo para o crescimento celular e invasão e diminuindo as respostas imunes do hospedeiro contra o tumor (Kaminska B, 2013). Vários estudos identificaram o TGF- β 1 como um biomarcador preditivo de cancro, com particular foco nos seus polimorfismos genéticos como os polimorfismos *TGF- β 1-509C/ T* e *869T/C* que, não conferindo suscetibilidade para o desenvolvimento de gliomas, podem ter impacto na sobrevivência dos doentes com GBM, especificamente, os genótipos *TGF- β 1-509TT* e *869CC* (Blobe GC, 2000; Elliott RL, 2005; Watanabe Y, 2002).

Além dos biomarcadores genéticos, o valor de vários metabolitos como biomarcadores de cancro foi amplamente investigado. São exemplo os lactatos e o rácio neutrófilo-linfócito. O lactato é um produto metabólico final de tumores altamente proliferativos, como GBM e resulta de um mecanismo de sobrevivência do tumor, que eflui ácido láctico para seu microambiente através de transportadores

transmembrana de monocarboxilato (Colen CB, 2011). Valores elevados dos lactatos séricos antes ou no início da cirurgia foram encontrados em doentes com gliomas de alto grau (Mariappan R, 2015; Shih CC, 2017). O rácio neutrófilo-linfócito (RNL) é um marcador hematológico de inflamação sistémica, havendo vários estudos a demonstrarem uma associação entre um RNL mais alto e um pior prognóstico em vários cancros. A inflamação associada ao cancro promove a angiogenese e a proliferação de células malignas, favorecendo a progressão da doença. Tem havido uma evidência crescente do RNL como factor independente de prognóstico nos doentes com glioblastoma (Mariappan R, 2015; Shih CC, 2017).

Montemurro N e col na revisão sistemática que efetuaram mostraram que há um conjunto de evidências de que a hiperglicemia e o índice de massa corporal (IMC) elevado são fatores de risco independentes para pior prognóstico e menor sobrevivência global nos doentes com GBM. Doentes com peso normal em comparação com obesos e doentes diabéticos medicados com metformina em comparação com outras terapias parecem ter uma sobrevivência global mais longa (Montemuro N, 2020). Também na investigação do nosso grupo, os doentes com glioblastoma e com excesso de peso e obesidade tiveram uma sobrevivência global mediana superior quando comparados com os doentes com peso normal (Aguilar PV, 2021).

OS FATORES IMAGIOLÓGICOS COM IMPORTÂNCIA PROGNÓSTICA

As características imagiológicas dos glioblastomas são outro aspecto atrativo para a determinação do prognóstico, uma vez que não carecem de pesquisa invasiva ou demorada podendo ser avaliadas de forma longitudinal no percurso da doença.

A presença, por exemplo, de componentes quísticos e de componentes necróticos no glioblastoma. Curtin L e col. mostraram, numa coorte de doentes com GBM que os que tinham lesões quísticas apresentavam uma sobrevivência global significativamente mais longa do que os com lesões não quísticas (Curtin L, 2020), o contrário acontecendo com os doentes que apresentavam uma elevada percentagem de componentes necróticos.

A pseudoprogressão é um fenómeno de alteração subaguda na imagem de ressonância magnética no glioma subsequente ao tratamento de radioquimioterapia sugestivo de progressão, com ou sem alterações clínicas associadas e que se resolve espontaneamente sem tratamentos adicionais. Os mecanismos patofisiológicos não estão completamente descritos, sendo sugerido que reflitam uma reação inflamatória do tecido após um tratamento local secundário a lesão vascular e oligodendrogliol, ou uma resposta exagerada a um tratamento eficaz (Linhares P, 2013). Algumas alterações genéticas e moleculares foram associadas à pseudoprogressão. Especificamente a sobreexpressão do p53, o estado de metilação do gene promotor do *MGMT*, o índice Ki67 e a mutação do *IDH1* foram relatados como biomarcadores potenciais para a deteção da pseudoprogressão (Linhares P, 2013).

O TRATAMENTO

O tratamento do glioblastoma é multimodal e engloba a cirurgia, a radioterapia (RT) nas suas diferentes vertentes e a terapia sistêmica como a quimioterapia (QT), as terapias dirigidas e imunoterapia. Algumas destas modalidades estão ainda em fase experimental e reservar-lhe-emos o benefício da eficácia. O primeiro tratamento e sempre que possível deve ser a cirurgia de ressecção máxima segura, um conceito que alia o benefício oncológico com o benefício funcional, seguida de RT (60 Gray [Gy] durante 6 semanas) com temozolamida (TMZ) diária concomitante seguida de 6 ciclos de manutenção com TMZ (Stupp R, 2005; Tan AC, 2020).

Na recidiva as opções terapêuticas não são tão consensuais e incluem a ressecção cirúrgica adicional, a re-irradiação, as terapias sistêmicas como lomustina ou bevacizumab, abordagens combinadas ou apenas cuidados de suporte (Tan AC, 2020). A imunoterapia pode ser ativa ou passiva (Tivnan A, 2017). A imunoterapia ativa tem como objectivo promover a resposta imunológica Th1 através de vacinas tumorais, vacinas celulares ou estimulantes imunológicos não-específicos. A imunoterapia passiva tem como objectivo induzir um efeito anti tumoral pela transferência de células imunes efectoras (Houang B, 2017). Incluiu os inibidores dos pontos de verificação imunológicos, a terapia de transferência adotiva de células, as vacinas terapêuticas e as terapias de vírus oncolíticos e a terapia direcionada que incluiu os inibidores da tirosina cinase e outros inibidores específicos de receptor, podendo ser potências tratamentos a explorar no glioblastoma (Khaddour K, 2020).

Três abordagens com vacinas chegaram aos ensaios de fase II e fase III. Uma vacina peptídica, Rindopepimut (ou CDX-110 ou PEPvIII), que mimetiza e consequentemente tem como alvo o EGFR vIII, que está expresso exclusivamente em células de glioblastoma em 25-30% dos doentes. A vacina multipeptídica ICT-107 que consiste em derivado de células dendríticas do doente incubadas ex-vivo com seis peptídeos de proteínas seleccionados com base em sua sobre-representação no perfil de expressão genético das células de glioblastoma em comparação com tecidos não malignos. Por último, a DCVax-L em que um lisado tumoral do glioblastoma ressecado do doente é usado para pulsar células dendríticas análogas e serve como uma fonte para todo o espectro de antígenos tumorais (Lim M, 2018).

Mais recentemente foi agregada à imunoterapia o tratamento com vírus oncolíticos, uma vez que atuam pela indução de uma resposta imunológica. Em maio de 2016, o poliovírus oncolítico recombinante PVSRIPO recebeu a designação de terapia

inovadora da FDA com base nas conclusões de um estudo de fase I em doentes com glioblastoma recorrente (Lim M, 2018).

A terapia celular adoptiva com *chimeric antigen receptor* (CAR) *T cells* baseia-se na potencialização dos linfócitos extraído do tumor, expandidos *in vitro* e reintroduzidos no doente. Na presença de IL-2 os linfócitos T são estimulados a destruir as células tumorais. CAR são recetores recombinantes que reconhecem antígenos de superfície celular, independentemente da expressão do HLA ou do processamento do antígeno. Assim, as células T geneticamente modificadas para expressar CARs apresentam especificidade de anticorpo para antígenos de superfície celular, permitindo que as células CART se fixem e destruam células tumorais que expressam as proteínas específicas (Shen SH, 2020). Ensaio clínico recente em doentes com glioblastoma avaliaram quatro alvos antigénicos: receptor $\alpha 2$ de IL-13 (IL-13R $\alpha 2$), receptor 2 do fator de crescimento epidérmico humano (HER2), receptor de carcinoma hepatocelular A2 (EphA2) produtor de eritropoietina e receptor do fator de crescimento epidérmico variante III (EGFRvIII) (Shen SH, 2020).

Por último surgiram como possibilidade terapêutica os inibidores dos pontos de verificação imunológicos (*immune-checkpoints*). Os recetores co-inibidores das células T desempenham um papel essencial na atenuação da força e da duração das respostas imunológicas mediadas pelas células T. Estes recetores inibitórios é que são referidos como moléculas de ponto de verificação imunológico e são responsáveis por manter a autotolerância e prevenir as reações autoimunes (Huang B, 2017). As duas moléculas co-inibidoras mais estudadas são o CTLA-4 (ou CD152) que atua no início da ativação das células T e o PD-1 que bloqueia as células T em fases posteriores da resposta imunológica. Os estudos focados na inibição da via PD-1/PDL1 mostraram resultados promissores.

O tratamento com campos elétricos alternados (*Tumor-treating fields* - TTF) é uma nova abordagem no glioblastoma. A adição desta modalidade ao regime TMZ/RT → TMZ (durante o período de manutenção TMZ) resulta numa melhoria da sobrevivência livre de progressão e da sobrevivência global nos doentes com glioblastoma recém-diagnosticado num estudo aberto de fase III (Lim M, 2018; Stupp R, 2017). O seu efeito na recidiva ainda está por demonstrar.

Mesmo com todos os avanços descritos, exceto os TTF, nenhuma outra intervenção terapêutica mostrou, de uma forma geral, prolongar a sobrevivência global no glioblastoma (Le Rhun E, 2019).

OS BIOMARCADORES

A identificação de biomarcadores que permitam aferir a existência de doença e monitorizar a evolução é crucial para a deteção precoce e pronto tratamento. O biomarcador ideal será uma molécula que possa ser detetada/quantificada por meio de métodos simples não invasivos ou minimamente invasivos com potencial para avaliar o risco de cancro, conseguir um diagnóstico precoce, aumentar a precisão da classificação, e monitorizar a evolução da doença e a resposta ao tratamento, além de estar fundamentalmente restrita apenas a um aspecto. Os biomarcadores do sangue periférico são particularmente atrativos pelo seu fácil acesso e têm sido amplamente utilizados para vários tipos de cancros. Um conjunto de biomarcadores séricos com múltiplas funções nos gliomas foram relatados como podendo classificar o grau do glioma com maior precisão e fornecer um valor prognóstico nesses doentes. No momento atual, o rastreio de gliomas não tem relevância clínica atendendo à baixa incidência, à falta de biomarcadores sensíveis no plasma e à observação de que os gliomas podem desenvolver-se de novo em algumas semanas ou meses. Até onde sabemos, não há uso rotineiro de um biomarcador sérico que ajude no diagnóstico ou no acompanhamento clínico. Marcadores baseados numa molécula única têm sido claramente insuficientes para acompanhar adequadamente a dinâmica de doenças como o cancro e devem ser substituídos por perfis de múltiplos marcadores.

OBJETIVOS

Com o conjunto de trabalhos presentes nesta dissertação e sabendo que:

1. Os factores mais importantes para o desenvolvimento dos gliomas são a proliferação neoplásica e neovascularização e que factores adicionais como a inflamação, a resposta imunológica e estado nutricional são também primordiais no desenvolvimento e progressão do glioblastoma;
2. Existem evidências do papel fulcral dos polimorfismos de genes reguladores destes dois mecanismos, na tumorigenese e resposta clínica;
3. A presença de células progenitores endoteliais (CPE) circulantes, assim como níveis séricos de outros factores de neovascularização, tem um papel importante no desenvolvimento dos tumores cerebrais.

Teve-se como objectivo:

1. Relacionar o fenómeno da pseudoprogressão com o prognóstico dos doentes com glioblastoma;
2. Correlacionar os achados genéticos determinados em sangue periférico com o diagnóstico e evolução clínica dos doentes;
3. Identificar biomarcadores em sangue periférico de susceptibilidade aumentada para o desenvolvimento de glioblastomas, assim como indicadores de prognóstico e resposta terapêutica nestes doentes.
4. Poder, eventualmente, servir de base para novas investigações em biomarcadores periféricos.

Para alcançar este objetivo foi de crucial importância o trabalho multidisciplinar amplo distribuído pelas diferentes instituições envolvidas e que culminou no desenvolvimento e publicação de 14 artigos pela equipa envolvida. Por uma questão de sistematização e fio condutor, faço nesta secção uma pequena descrição dos trabalhos, colocando neste manuscrito apenas os que sou primeiro autor. Os restantes artigos seguirão no Anexo 1, sendo, contudo, alvo de discussão no respetivo capítulo.

1. Relacionar o fenómeno da pseudoprogressão com o prognóstico dos doentes com glioblastoma

a. Dados imagiológicos como biomarcador de prognóstico

A abundância de biomarcadores de sangue periférico descritos na literatura, com resultados pouco conclusivos e, muitas vezes, contraditórios levou-nos a procurar dados em áreas paralelas. A realização sistemática e universal de ressonância magnética para seguimento dos doentes com glioblastoma tornava-a num objeto de estudo facilmente acessível. Começava a falar-se na pseudoprogressão como

resposta cerebral à agressão terapêutica levantando-se a hipótese que uma maior reação se traduzia numa melhor resposta. Nesse sentido fomos avaliar de que forma a resposta inflamatória traduzida em imagem de ressonância magnética poderia servir de marcador de prognóstico. Para isso fomos ver se os critérios usados para avaliação imagiológica dos doentes submetidos a tratamento de radioterapia e quimioterapia eram adequados para avaliação e se a presença duma resposta inflamatória mais marcada na ressonância seria um factor de prognóstico nestes doentes. Assim, comparamos os critérios de MacDonald com os critérios de *Response Assessment in Neurooncology* (RANO) para avaliar a sua concordância e tentar perceber se seriam necessários novos parâmetros de avaliação (Artigo 1).

2. Correlacionar os achados genéticos determinados em sangue periférico com o diagnóstico e evolução clínica dos doentes.

a. Estudo dos polimorfismos do VEGF

Para a correlação dos achados genéticos determinados em sangue periférico com o diagnóstico e a evolução clínica dos doentes, começamos por estudar os polimorfismos do gene *VEGF* na determinação do risco de desenvolvimento e prognóstico nos doentes com glioblastoma. Fizemos um estudo caso-controlo com 126 doentes com glioma e 180 controlos saudáveis. Foram estudados 11 polimorfismos do gene *VEGF*. Para isto foi extraído o ADN dos leucócitos do sangue periférico e procedeu-se à genotipagem dos produtos de extensão de *primer* específicos de alelo, que foram gerados a partir das sequências de ADN amplificadas (Artigo 2).

b. Estudo dos polimorfismos rs4977756 (*CDKN2A/B*), rs6010620 (*RTEL*), rs498872 (*PHLDB1*), rs2736100 (*TERT*) e rs4295627 (*CCDC26*)

Com o avanço dos estudos de polimorfismos de nucleotídeo único e a constatação que estes SNPs são um dos os tipos mais comuns de variação genética no genoma humano e estão envolvidos na etiologia e suscetibilidade do cancro e pela identificação em estudos de associação genómica alargada (GWAS) de 5 polimorfismos associados com o risco de desenvolvimento de glioblastoma, o nosso grupo tentou validar estes achados na nossa população, tendo realizado um estudo caso-controlo dos SNPs rs4977756 (*CDKN2A/B*), rs6010620 (*RTEL*), rs498872 (*PHLDB1*), rs2736100 (*TERT*) e rs4295627 (*CCDC26*) (Artigo 1 – Anexo 1).

c. Estudo dos polimorfismos do EGFR

Para a determinação do prognóstico dos glioblastomas o nosso grupo tinha inicialmente procurado o impacto dos polimorfismos do *EGFR*, tendo realizado um estudo caso-controlo com 196 doentes com glioma e 168 controlos saudáveis, tendo sido feita a genotipagem *EGFR* 216G/T e 191C/A por amplificação PCR da região promotora dos nucleotídeos 352 a 155 seguida por RFLP (Artigo 2 - Anexo 1).

d. Estudo dos polimorfismos do *TGF-β*

Sabendo que a carcinogénese envolve múltiplos mecanismos e diferentes genes, sendo o factor de crescimento tumoral beta um dos agentes envolvidos em muitos cancros, fomos avaliar a relevância dos polimorfismos -509C/T e 869T/C do *TGF-β*1 no risco de glioma e no seu prognóstico. Para isso, o nosso grupo efetuou um estudo caso-controlo com 114 doentes com glioma e 138 controlos saudáveis. O ADN genómico dos casos de glioma e controlos foi extraído de leucócitos de sangue periférico por tratamento proteinase K/clorofórmio/isopropanol. O DNA purificado foi usado para determinar os genótipos de ambos os polimorfismos, usando o método da reação em cadeia da polimerase seguida por do polimorfismo de restrição de comprimento de fragmento (PCR-RFLP) (Artigo 3 – Anexo 1).

e. Estudo dos polimorfismos do HOTAIR

Os polimorfismos de nucleotídeo único do gene *HOX Transcript Antisense* RNA (HOTAIR) rs920778 (C>T) e rs12826786 (C>T) presentes no intensificador intronico e regiões promotoras do HOTAIR, respetivamente, estão associados com a suscetibilidade ao cancro e com o prognóstico de doentes com alguns tipos de tumor. Nesse sentido, o nosso grupo foi avaliar a relevância deste achado nos doentes com glioblastoma. Para isso realizou um estudo de caso-controlo com 177 doentes com glioma e 199 controlos saudáveis. Todos os indivíduos foram genotipados por PCR e polimorfismo de comprimento de fragmento de restrição (RFLP) (Artigo 4 – Anexo 1).

f. Estudo de genes de imunoglobulina implicados no risco de glioma

Os fatores genéticos e ambientais são considerados causais na gliomagenesis. Vários genes foram implicados no desenvolvimento de glioma, mas o papel putativo de um importante membro do complexo genético relacionado com a imunidade, a imunoglobulina de cadeia pesada γ (IGHG) não tinha sido avaliada. O nosso grupo para testar essa hipótese, genotipou o locus IGHG que compreende os alelos GM, especificamente os alelos GM 3 e 17, de 120 doentes com glioma e 133 controlos saudáveis (Artigo 5 – Anexo 1).

3. Identificar biomarcadores em sangue periférico de susceptibilidade aumentada para o desenvolvimento de glioblastomas, assim como indicadores de prognóstico e resposta terapêutica nestes doentes.

a. Influência do rácio neutrófilo-linfócito no prognóstico dos doentes com glioblastoma

O rácio neutrófilos-linfócitos (NLR) é um marcador hematológico de inflamação sistémica e vários estudos demonstraram uma associação entre um NLR mais alto e um pior prognóstico em várias doenças malignas. Esta relação não estava cabalmente demonstrada nos doentes com glioblastoma. Assim, fomos avaliar

retrospectivamente uma base de dados prospetiva de doentes com glioblastoma submetidos a remoção cirúrgica, tendo incluído 140 doentes. Fomos estudar os valores de neutrófilos, de linfócitos e de plaquetas de colheitas de sangue periférico realizadas antes da cirurgia, nas 24 horas pós-operatórias e na primeira consulta antes do início do tratamento adjuvante de radio e quimioterapia (Artigo 12 – Anexo 1). Há medida que foram saindo resultados a corroborar os nossos achados, reforçamos a importância deste índice numa letter ao editor, como comentário, realçando a ideia de que estes resultados não são absolutos e que podem apenas ser representativos de um subgrupo específico de doentes. (Artigo 3).

b. Relação do valor dos lactatos séricos com o grau de malignidade dos gliomas

A crescente necessidade da identificação de biomarcadores de fácil acesso e monitorização levou-nos a procurar metabolitos que traduzissem o estado proliferativo dos gliomas. Os lactatos são um produto metabólico final de tumores altamente proliferativos e resultam de um mecanismo de sobrevivência do tumor, que eflui o ácido láctico para o seu microambiente através de transportadores transmembrana de monocarboxilato. A inibição da função destes transportadores compromete a invasão dos gliomas. Reparamos que um número significativo de doentes com glioblastoma apresentava níveis séricos de lactatos aumentados antes da cirurgia. Assim, fomos rever os dados de 152 doentes submetidos a remoção cirúrgica ou biópsia de gliomas e avaliamos a relação entre o valor dos lactatos séricos com o grau histológico do glioma (Artigo 4).

c. Associação entre o RDW e a sobrevivência nos doentes com glioblastoma

O índice de anisocitose eritrocitária (*Red cell distribution width* - RDW) é um marcador celular sistémico de inflamação e foi descrito como um possível indicador de prognóstico nalguns tipos tumorais, sendo que no glioblastoma estava pouco estudado. Considerando que a sua avaliação faz parte do estudo sanguíneo pré-operatório básico, torna-se uma variável de fácil acesso e universal. Estudos prévios apresentam resultados dispares em relação ao papel potencial do RDW como um fator de risco independente para a sobrevivência nos doentes com glioblastoma. Por esse motivo fomos avaliar o RDW como um fator prognóstico de progressão da doença e sobrevivência global numa coorte de doentes com glioblastoma submetidos a ressecção cirúrgica. Avaliamos 140 doentes, 98 homens e 42 mulheres, com média de idade de $62,9 \pm 10,00$ anos tentando determinar o papel do RDW no prognóstico e sobrevivência global destes doentes (Artigo 5).

d. Existirá algum biomarcador de sangue periférico com verdadeira relevância clínica?

Com a falência de resultados francamente conclusivos que mostrassem que um biomarcador isolado permitisse fazer o diagnóstico e o seguimento dos doentes com

glioblastoma, fomos procurar na literatura se a orientação que estávamos a dar aos trabalhos seria a mais adequada ou se teríamos que redirecionar a orientação da investigação. Nesse sentido fizemos uma busca exaustiva da literatura foi realizada usando os bancos de dados *MEDLINE* e *Cochrane* desde janeiro de 2005 a dezembro de 2018 usando os termos “glioma, biomarcadores séricos”. Consideramos apenas os artigos em inglês e os estudos sobre dados coletados em humanos. Dividimos os biomarcadores em categorias específicas, embora vários deles tenham várias funções e essas categorias sejam altamente inter-relacionadas: (a) crescimento celular e proliferação vascular; (b) imune sistema; (c) inflamação; (d) estado nutricional; e (e) marcadores de coagulação (Artigo 6).

4. Eventualmente servir de base para novas investigações em biomarcadores periféricos.

Iniciamos um estudo piloto para pesquisa de alterações do DNA circulante dos doentes com glioblastoma. Este projeto está na fase de início tendo sido obtido colheitas de sangue periférico em 13 doentes e colheita de tecido tumoral em 7 doentes.

Iniciamos um projeto de investigação clínica que consiste num estudo prospetivo da avaliação sequencial dos valores hematológicos como fator de prognóstico nos doentes com glioblastoma.

Iniciamos um projeto de investigação clínica que consiste num estudo prospetivo da pseudoprogressão como fator de prognóstico nos doentes com glioblastoma.

E pretendemos fazer uma mudança de paradigma em termos de investigação de translação – em vez de estudar o que corre bem, estudar o que corre mal, ou seja, avaliar as características genéticas, moleculares e clínicas dos doentes com glioblastoma “*short survivors*” e compará-las com os “*normal*” e os “*long survivors*”.

ARTIGOS PUBLICADOS

1 EARLY PSEUDOPROGRESSION FOLLOWING CHEMORADIOOTHERAPY IN GLIOBLASTOMA PATIENTS: THE VALUE OF RANO EVALUATION

Paulo Linhares,^{1,2} Bruno Carvalho,^{1,2} Rita Figueiredo,³ Rui M. Reis,^{4,5} and Rui Vaz^{1,2}

¹Department of Neurosurgery, Hospital de São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal;

²Faculty of Medicine, University of Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal; ³ Department of Neuroradiology, Hospital de São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal; ⁴ Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; ⁵Molecular Oncology Research Center, Barretos Cancer Hospital, Rua Antenor Duarte Villella, 1331 Bairro Dr. Paulo Prata, Barretos 14784-400, SP, Brazil.

Clinical Study

Early Pseudoprogression following Chemoradiotherapy in Glioblastoma Patients: The Value of RANO Evaluation

Paulo Linhares,^{1,2} Bruno Carvalho,^{1,2} Rita Figueiredo,³ Rui M. Reis,^{4,5} and Rui Vaz^{1,2}

¹ Department of Neurosurgery, Hospital de São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

² Faculty of Medicine, University of Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal

³ Department of Neuroradiology, Hospital de São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

⁴ Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

⁵ Molecular Oncology Research Center, Barretos Cancer Hospital, Rua Antenor Duarte Vilella, 1331 Bairro Dr. Paulo Prata, Barretos 14784-400, SP, Brazil

Correspondence should be addressed to Bruno Carvalho; bmfcarvalho@gmail.com

Received 1 May 2013; Revised 10 July 2013; Accepted 11 July 2013

Academic Editor: Minesh P. Mehta

Copyright © 2013 Paulo Linhares et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction. The aim of this study was to determine the frequency of pseudoprogression in a cohort of glioblastoma (GBM) patients following radiotherapy/temozolomide (RT/TMZ) by comparing Macdonald criteria to Response Assessment in Neuro-Oncology (RANO) criteria. The impact on prognosis and survival analysis was also studied. **Materials and Methods.** All patients receiving RT/TMZ for newly diagnosed GBM from January 2005 to December 2009 were retrospectively evaluated, and demographic, clinical, radiographic, treatment, and survival data were reviewed. Updated RANO criteria were used for the evaluation of the pre-RT and post-RT MRI and compared to classic Macdonald criteria. Survival data was evaluated using the Kaplan-Meier and log-rank analysis. **Results and Discussion.** 70 patients were available for full radiological response assessment. Early progression was confirmed in 42 patients (60%) according to Macdonald criteria and 15 patients (21%) according to RANO criteria. Pseudoprogression was identified in 10 (23.8%) or 2 (13.3%) patients in Macdonald and RANO groups, respectively. Cumulative survival of pseudoprogression group was higher than that of true progression group and not statistically different from the non-progressive disease group. **Conclusion.** In this cohort, the frequency of pseudoprogression varied between 13% and 24%, being overdiagnosed by older Macdonald criteria, which emphasizes the importance of RANO criteria and new radiological biomarkers for correct response evaluation.

1. Introduction

The current standard of care for newly diagnosed glioblastoma (GBM) remains surgical resection followed by radiotherapy with concurrent temozolomide (RT/TMZ) and then maintenance temozolomide for at least six months [1]. The addition of chemotherapy introduced new questions regarding biological response of the tumor and the surrounding brain. Pseudoprogression is a phenomenon of subacute imaging changes in human glioma subsequent to radiochemotherapy suggestive of progression, with or without associated clinical sequelae, which resolve spontaneously without further therapy [2]. It was first reported by Hoffman et al. [3]

in 1979 and fully described by de Wit et al. [4] in 2004. The underlying pathophysiological mechanisms are not fully understood [5]. Some authors suggest that it likely reflects an inflammatory tissue reaction after a local treatment secondary to vascular and oligodendroglial injury, whereas others associate it with an exaggerated response to effective therapy [2, 6, 7].

The incidence of pseudoprogression, time of occurrence, and its impact on prognosis remain poorly defined. Incidence has been reported to vary between 12% and 64% in different series, and this phenomenon commonly occurs in the first 3 months after radiochemotherapy, occasionally persisting for up to six months after treatment [2, 5] Imaging changes

suggestive of pseudoprogression consist of an increase in gadolinium enhancement on T1-weighted images and peritumoral edema [5, 8]. Despite technical advances in perfusion magnetic resonance imaging (MRI) and positron emission tomography (PET), no imaging technique is yet validated for detection of pseudoprogression and has not been incorporated into the latest response assessment criteria [9–11].

Genetic and molecular changes have also been associated with pseudoprogression. Specifically p53 overexpression [12], O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation status [13], Ki67 indices [14], and isocitrate dehydrogenase 1 (IDH1) mutation have been reported as potential biomarkers for detecting pseudoprogression [15].

The response of brain tumors is classically reported using Macdonald criteria based on the two-dimensional measurement of the enhancing area on CT or MRI, in conjunction with clinical assessment and corticosteroid dose [6]. However, this response evaluation has important limitations since posttreatment contrast enhancement in brain tumors is non-specific and may not always be considered as a true surrogate of tumor response [16]. Differentiation between pseudoprogression and real progression is critical for making decisions on future therapy and for predicting the prognosis in clinical practice [17].

The purpose of this study was to clarify the frequency of pseudoprogression after radiochemotherapy in a cohort of Portuguese patients with glioblastoma, by comparing RANO with the classical Macdonald evaluation.

2. Materials and Methods

One hundred seventeen adult patients with newly diagnosed glioblastoma were treated between January 2005 and December 2009 with radiochemotherapy according to the Stupp protocol. Seventy patients were available for complete radiological response assessment.

All patients were retrospectively evaluated, and demographic, clinical, radiographic, treatment, and survival data were reviewed. The inclusion criteria were age ≥ 18 years with histologically proved glioblastoma and the availability of MRI imaging less than 72 hours postoperatively and at each 3 months to define response. Response assessment was independently performed by a neuroradiologist (RF), according to RANO criteria [11]. These data were subsequently compared to Macdonald criteria evaluation [18]. Table 1 summarizes the response assessment criteria as adapted from Wen et al. [11].

Pseudoprogression was defined as a 25% increase in tumor size or any new lesion followed by stabilization (stable disease) or response (partial or complete), with no therapy modification, for at least 6 months after completing the concomitant phase of the RT/TMZ [19]. According to RANO criteria, progression within <12 weeks after completion of chemoradiotherapy was only defined if there was new enhancement outside of the radiation field (beyond the high-dose region or 80% isodose line) or if there was unequivocal evidence of viable tumor on histopathologic sampling. So, given the difficulty of differentiating true progression from pseudoprogression, clinical decline alone as well as an

increase of contrast enhancement within the radiotherapy field was not considered sufficient for definition of progressive disease within the first 12 weeks after completion of concurrent chemoradiotherapy [11].

Demographic data are collected and summarized in Table 2.

Temozolomide was administered at a dose of 75 mg per m^2 concurrent with daily radiotherapy and followed by 150–200 mg per m^2 for 5 days every 28 days. Radiation was delivered in 2 Gy/fr, for a total of 60 Gy in 30 fractions. Patients were first categorized as having early progression if there was progression at the first follow-up scan 1 to 3 months after chemoradiotherapy and no early progression if there was stable or responsive disease. Patients with early progression were further subdivided in pseudoprogression and true progression according to further radiological progression.

2.1. Statistics. The primary endpoint was the frequency of pseudoprogression and its effect on prognosis. The secondary endpoints were overall survival and progression-free survival calculated from the date of surgery. Kaplan-Meier product-limit analysis was conducted and 95% confidence intervals were calculated. Log-rank test was used to detect statistically significant differences in survival distributions. Data of patients who have not progressed or died were right censored in our analysis. Multivariate survival analysis using a forward stepwise Cox regression model was used to determine independent predictors of survival. Borderline significant variables ($P \leq 0.1$) were included in the final model. SPSS software version 19.0 was used for the statistical analysis.

3. Results

At the first MRI scan performed 1 month after concurrent RT/TMZ, early progression was recorded in 42 patients (60%), while 28 patients were considered to have nonprogressive disease (nPD), according to Macdonald criteria. Thirty-four patients (81%) met the Macdonald criteria for progression due to a 25% increase in tumor size, 4 patients (9.5%) due to new lesions and 4 patients (9.5%) due to clinical deterioration. Out of the 42 patients with early progression there were 32 patients (76%) with further progression (tPD) and 10 (24%) patients with stable or reduced lesion—pseudoprogression (psPD) (Figure 1). Regarding nPD patients, 26 patients (93%) had stable disease and 2 patients (7%) showed partial response during subsequent follow-up.

When considering RANO criteria for progression within the first 12 weeks after completion of concurrent chemoradiotherapy, there were only 15 patients (21%) who met the criteria for progressive disease, while 55 patients (79%) were considered to have nonprogressive disease, either stable disease (27 patients), partial response (1 patient) or an increase of contrast enhancement within the radiotherapy field (27 patients), also considered stable disease. Out of the 15 early progressive patients, there were 14 patients with new enhancement areas outside of the radiation field and 1 patient with evidence of viable tumor on histopathologic sampling. Subsequently, 13 patients (87%) showed further progression

TABLE 1: Criteria for response assessment in high-grade gliomas.

Response	Criteria	
	Macdonald criteria	RANO criteria
Complete response	All: complete disappearance of all enhancing measurable and nonmeasurable diseases sustained for at least 4 weeks, no new lesions, no corticosteroids, and being stable or improved clinically.	All: T1 gadolinium enhancing disease, none; T2/FLAIR, stable or decreasing; new lesion, none; corticosteroids, none; clinical status: stable or improving.
Partial response	All: $\geq 50\%$ decrease in sum of products of perpendicular diameters of all measurable enhancing lesions sustained for at least 4 weeks, no new lesions, stable or reduced corticosteroid dose, and being stable or improved clinically.	All: T1 gadolinium enhancing disease, $\geq 50\%$ decrease; T2/FLAIR, stable or decreasing; new lesion, none; corticosteroids, stable or decreasing; clinical status: stable or improving.
Stable disease	All: being not qualified for complete response, partial response, or progression; being stable clinically.	All: T1 gadolinium enhancing disease: $< 50\%$ decrease but $< 25\%$ increase; T2/FLAIR: stable or decreasing; new lesion: none; corticosteroids: stable or decreasing; clinical status: stable or improving.
Progression	Any: $\geq 25\%$ increase in sum of the products of perpendicular diameters of enhancing lesions, any new lesion, or clinical deterioration.	Any: T1 gadolinium enhancing disease: $\geq 25\%$ increase; T2/FLAIR: increasing; new lesion: none; corticosteroids: not applicable; clinical status: deteriorating.

RANO: Response Assessment in Neuro-Oncology; FLAIR: fluid-attenuated inversion recovery.

TABLE 2: Demographic data of population.

Characteristics	Patients	
	Number	%
Age, years		
Median	62	
Range	34–78	
Gender		
Male	46	66
Female	24	34
ECOG		
0	18	25.7
1-2	43	61.4
3	9	12.9
Surgery		
Biopsy	14	20
Partial resection	32	46
Total resection	24	34
Adjuvant TMZ cycles		
Mean	5	
Range	0–20	
Corticotherapy		
Stable	43	61
Increased	11	16
Reduced	16	23

(tPD) and 2 patients (13%) showed stable disease representing pseudoprogression (psPD).

In fact, RANO criteria were specifically structured to make overcalling of early progression in the first 3 months difficult, so, as expected, progressive disease < 12 weeks as assessed by RANO criteria revealed a lower value.

After progressive disease in the first MRI 1 month after concurrent RT/TMZ, we observed dissociated responses

between T2/FLAIR and T1-contrast-enhanced imaging in 3 patients in whom stabilization or decreasing enhancement was accompanied by an increase in T2/FLAIR signal without any modification in therapy (Figure 2). Subsequent imaging confirmed progression.

Regarding corticosteroid therapy in psPD group, 5 patients showed stable dose, 4 had decreasing doses, and only 1 had transient dose increment.

Functional status remained stable in patients with pseudoprogression according to the ECOG performance status.

3.1. Survival Analysis. The whole cohort median survival and median progression-free survival were 15 months [95% CI (13.64, 16.36)] and 10 months [95% CI (8.02, 11.98)], respectively. The only independent predictors of survival were the number of TMZ cycles and the type of surgery (Table 3). Interestingly, multivariate analysis showed that the number of TMZ cycles was a strong predictor for survival regardless of the ECOG status.

Survival analysis for subgroups of progression according to Macdonald criteria revealed a median overall survival of 12 months [95% CI (8.7, 15.3)] for the group of true progressive disease and 21 months [CI 95% (14.5, 27.5)] for the group of nonprogressive disease. Pseudoprogression group had a median overall survival of 24 months [CI 95% (11.6, 36.4)], not statistically different from the nonprogressive group ($P = 0.456$). According to RANO criteria, true progressive disease group had a median overall survival of 9 months [95% CI (3.7, 14.3)] and nonprogressive disease group had a median overall survival of 16 months [95% CI (13.8, 18.2)]. Pseudoprogression group was limited to 2 patients with an estimate median overall survival of 13 months, not statistically different from the nonprogressive group ($P = 0.639$) (Figure 3). Median progression-free survival for true progressive disease, pseudoprogression, and nonprogressive disease was 6 months, 16 months, and 12 months, respectively (Macdonald criteria

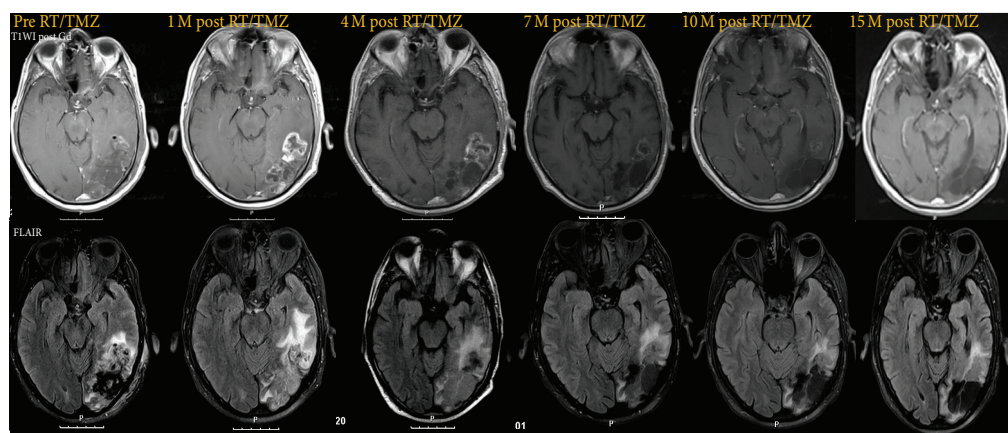


FIGURE 1: T1-contrast-enhanced and FLAIR magnetic resonance imaging (MRI) documenting pseudoprogression. Compared with pre-RT/TMZ MRI, the 1-month post-RT/TMZ showed increasing areas of contrast enhancement suggestive of tumoral progression. The patient remained clinically stable with stable dose of corticotherapy. Adjuvant TMZ treatment cycles were maintained and serial MRI at 4, 7, 10, and 15 months showed a consistent reduction in the size and the contrast of the lesion as well as in FLAIR signal.

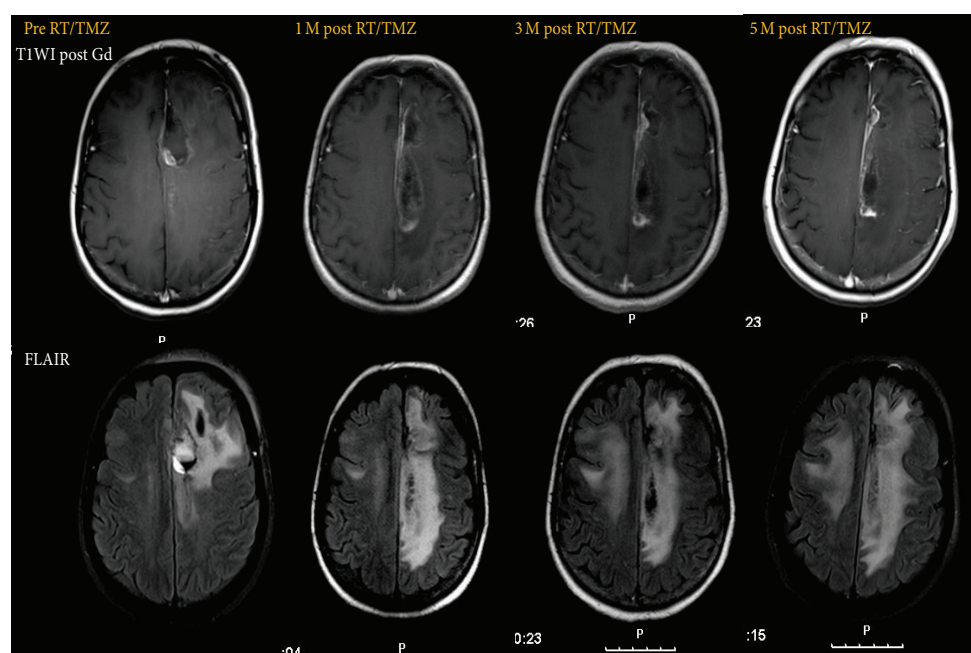


FIGURE 2: T1-contrast-enhanced magnetic resonance imaging (MRI) documenting pseudoprogression according to Macdonald criteria showing a significant increase in cingulate gyrus enhancing lesion at 1 month after RT/TMZ with subsequent stabilization (stable corticosteroid dose and functional status). FLAIR sequence shows increased signal extending to contralateral hemisphere anticipating true progressive disease.

applied) and 6 months, 7 months, and 11 months, respectively (RANO criteria applied) (Figure 4).

Comparing the survival distributions, patients with pseudoprogression showed a statistically significant improved overall survival over patients with true progressive disease

($P = 0.01$) and an increased time to progression ($P < 0.001$), according to Macdonald response assessment. Survival curves of psPD and nPD closely overlap showing no statistical difference ($P = 0.456$). Regarding RANO assessment group, the low rate of pseudoprogression (2/15 patients, 13%)

TABLE 3: Analysis of independent predictors of survival in the cohort of glioblastoma patients submitted to chemoradiotherapy.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age						
≥60 versus <60 years	1.26	0.75–2.10	0.389	—	—	—
Gender						
M versus F	0.98	0.57–1.69	0.943	—	—	—
Type of surgery						
Biopsy versus total or subtotal resection	3.47	1.83–6.58	<0.001	2.60	1.35–4.99	0.004
Number of surgeries						
1 versus >1	0.97	0.54–1.75	0.921	—	—	—
Corticosteroids						
Increasing versus stable or decreasing	1.04	0.54–2.02	0.903	—	—	—
ECOG						
0–2 versus >2	0.34	0.17–0.72	0.005	ns		
Number of TMZ cycles						
≥6 versus <6	0.16	0.09–0.29	<0.001	0.18	0.10–0.32	<0.001

CI: confidence interval; HR: hazard ratio; M: male; F: female; TMZ: temozolomide; ECOG: Eastern Cooperative Oncology Group; ns: nonsignificant. Results indicated in bold are significant. Multivariate analysis used Cox proportional hazards model with a forward stepwise regression procedure.

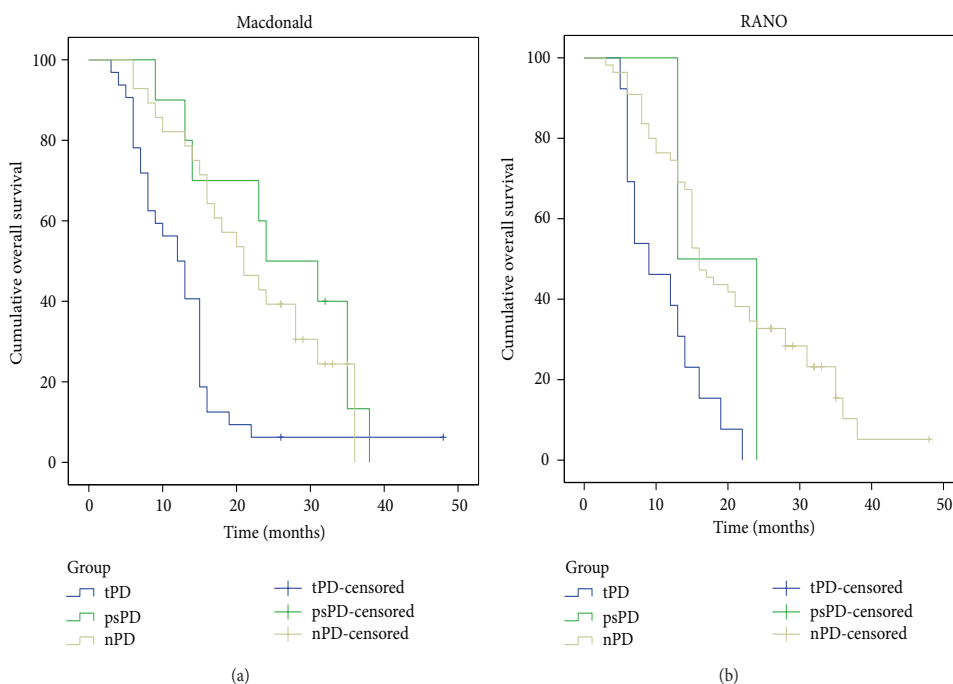


FIGURE 3: Kaplan-Meier analysis of *overall survival* curves according to different progression groups: true-progressive disease (tPD), blue; Pseudoprogression (psPD), green; Nonprogressive disease (nPD), yellow; and different response assessment criteria: (a), Macdonald; (b), RANO.

did not provide enough statistical power to allow significant statistical conclusions in this particular group.

4. Discussion

Pseudoprogression is characteristically found within 2 to 3 months after treatment but can occur at 6 months and may

progress over time into treatment-related necrosis [17, 31]. The issue of contrast enhancement areas starts even before adjuvant therapy administration. Farace et al. studied early MRI changes in the period between surgery and adjuvant therapy by diffusion-weighted imaging and MR perfusion, concluding that areas of new contrast enhancement of resected GBM are frequently observed in this period (17/37,

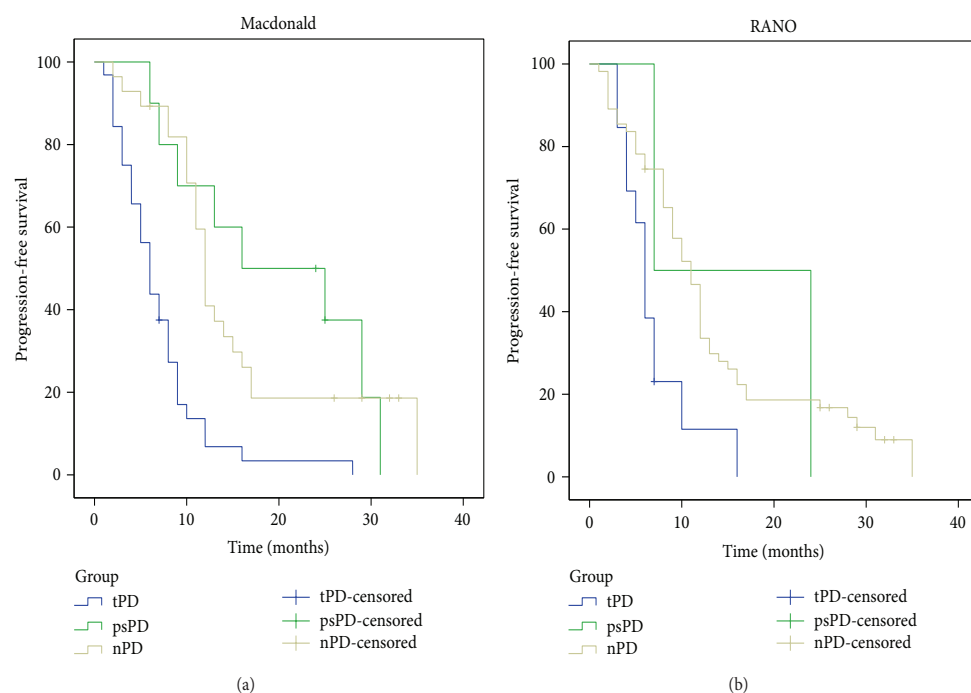


FIGURE 4: Kaplan-Meier analysis of *progression-free survival* curves according to different progression groups: true-progressive disease (tPD), blue; pseudoprogression (psPD), green; nonprogressive disease (nPD), yellow; and different response assessment criteria: (a) Macdonald and (b) RANO.

46%) and that most of them are suggestive of tumor progression (14/17; 82%) [32]. Distinguishing treatment induced imaging changes from true progressive disease can be especially difficult in the period immediately following completion of radiotherapy [8]. Standard MR imaging techniques do not allow a clear distinction between recurrent tumour and radiation-induced lesion [33]. The MRI characteristics of pseudoprogression have been described as an asymptomatic increase in contrast enhancement [34]. Enhancement of brain tumors primarily reflects a disturbed blood-brain barrier, and all the processes that decrease or increase this abnormal permeability will affect the area of enhancement, regardless of the size and activity of the tumor [31]. According to the literature, pseudoprogression occurs in 12 to 64% of patients. Table 4, as adapted from Kruser et al., summarizes the data [5, 8, 10, 12–14, 17, 19–29].

Topkan et al. evaluated pathological confirmed incidence of pseudoprogression in a cohort of 63 patients. Pseudoprogression was identified in 12 out of 28 patients with early progressive disease (42.8%), representing an overall pseudoprogression rate of 19% [30]. The wide range of variation in retrospective cohorts might be explained by different criteria used to establish the response and, also, by considering different time periods. We only considered early progression in the first 3 months, and by this we could have lost some progressive patients. Our rates of 13 to 24% of pseudoprogression are in line with the literature. We retrospectively reviewed

RANO criteria and compared them to Macdonald criteria since the latter do not take into account progressive nonenhancing disease, disregarding enlarging areas of nonenhancing tumor as evidence of tumor progression. Recently, a study comparing several response assessment criteria in recurrent GBM concluded a strong concordance between methods but noticed that criteria integrating FLAIR hyperintensity tended to reduce response rates and progression-free survival compared with criteria considering only contrast enhancement [35]. The decreased rate of pseudoprogression in our data after RANO evaluation reflects the improvement in response assessment. In fact, as discussed elsewhere, tumor progression as determined by both contrast-enhanced T1 and T2 sequences is more frequently diagnosed than when considering only contrast-enhanced T1 sequences [36]. Currently, changes in T2/FLAIR are not based on objective or volumetric measures due to technical limitations and insufficient standardization but should include mass effect and infiltration of the cortical ribbon or location outside of the radiation field [11].

The abnormal enhancement seen in pseudoprogression may be the result of treatment-related cellular hypoxia, which results in expression of hypoxia-regulated molecules from tumor and surrounding cells with the subsequent increased vascular permeability/increased tumor enhancement [7]. Pseudoprogression likely involves early changes to the vascular endothelium and the blood-brain-barrier [5]. It may be

TABLE 4: Rates of pseudoprogression reported in the literature in patients treated with radiotherapy and temozolomide.

Author	Year	Number of patients	Response criteria	Period of early-response assessment (months)	Number with ePD	psPD (% of ePD)	Overall rate of psPD
Chamberlain et al. [20]	2007	51	Not specified	6	26	7/15 (47%)	7/40 (18%)
Brandes et al. [13]	2008	103	Macdonald	1	50	32/50 (64%)	32/103 (31%)
Taal et al. [19]	2008	68	Macdonald	1	31	15/31 (48%)	15/68 (22%)
Chaskis et al. [21]	2009	54	Gd-MRI + clinical	6	25	3/12 (12%)	3/54 (6%)
Clarke and Chang [22]	2009	85	Macdonald	0.5–1	35	10/27 (37%)	10/77 (13%)
Fabi et al. [23]	2009	12	Macdonald	2	4	2/4 (50%)	2/12 (17%)
Peca et al. [24]	2009	50	Gd-MRI + MR Spectroscopy	6	15	4/15 (27%)	4/50 (8%)
Roldan et al. [8]	2009	43	Macdonald	1–1.5	25	10/20 (50%)	10/38 (26%)
Gerstner et al. [25]	2009	45	Macdonald	0.5–1	24	13/24 (54%)	13/45 (29%)
Sanghera et al. [5]	2010	104	RECIST	2	27	7/22 (32%)	7/99 (7%)
Tsien et al. [10]	2010	27	Macdonald	3	14	6/14 (43%)	6/27 (22%)
Yaman et al. [26]	2010	67	Gd-MRI + T2/FLAIR	6	17	4/17 (24%)	4/67 (6%)
Gunjur et al. [27]	2011	68	Macdonald	1	41	14/41 (34%)	14/68 (21%)
Kang et al. [12]	2011	35	Macdonald	1	18	8/18 (44%)	8/35 (23%)
Kong et al. [17]	2011	90	Macdonald + rCBV	2	59	26/59 (44%)	26/90 (29%)
Young et al. [28]	2011	321	Macdonald	0.5–1	205	30/93 (32%)	Not specified
Park et al. [29]	2011	48	Macdonald	1	25	11/25 (44%)	11/48 (23%)
Topkan et al. [30]	2012	63	Macdonald	6	28	12/28 (43%)	12/63 (19%)
Pouveau et al. [14]	2012	63	Gd-MRI + edema	2	33	7/33 (21%)	7/63 (11%)
Present study	2013	70	RANO	3	15	2/15 (13%)	2/70 (3%)
			Macdonald		42	10/42 (24%)	10/70 (14%)

ePD: early progressive disease; tPD: true progressive disease; psPD: pseudoprogression; Gd-MRI: gadolinium enhanced-MRI.

considered as a pathological continuum between acute post-RT reaction and treatment-related necrosis. More recently, Kruser et al. in their comprehensive review highlight the dichotomy between pseudoprogression—as a clinical diagnosis in the absence of intervention, and RT necrosis which can only be defined upon reoperation or biopsy [37]. Mechanisms behind these events are not fully understood but are probably related to microvessels changes. Post-RT brain injury induces a focal tissue reaction with inflammatory component and increased capillary permeability, causing fluid transudation into the interstitial space and brain oedema. Transient alterations in the blood-brain barrier may be responsible for new or increased contrast enhancement, falsely suggesting tumour progression, with low perfusion values [33].

There is also evidence that treatment-related necrosis occurs more frequently and earlier after temozolomide chemotherapy than after radiotherapy alone [31]. The MRI characteristics of pseudoprogression are similar to those of radiation necrosis, and conventional sequences are unable to distinguish between the two [28]. Corpus callosum involvement, subependymal spread, or multiple enhancing lesions are more consistent with progressive disease than pseudoprogression but are not able to reliably discriminate between both responses [28, 38]. Additionally, nonenhanced tumor is

usually not considered in response evaluation as shown in the several retrospective studies. This represents a strong limitation in response assessment. Some advanced MRI techniques have shown promising results of distinguishing posttreatment necrosis from tumor progression and pseudoprogression, including MR spectroscopy (higher Cho/Cr and Cho/NAA ratios), MR perfusion imaging, and diffusion-weighted MRI, namely, relative cerebral blood volume (rCBV) as an imaging biomarker for increased neoangiogenesis and apparent diffusion coefficient (ADC) value as an imaging biomarker of increased cellular density [10, 17, 38–41].

Our data are in agreement with the literature as we have noticed an increase in overall survival and in time to progression in patients with pseudoprogression compared to patients with true progression as assessed by classical criteria. On the other hand, our results show no differences between the pseudoprogression patients and the patients with nonprogressive disease in contrast to some authors who report survival benefit in pseudoprogression patients [13] although the small number of patients enrolled in our study may preclude conclusions on the significance of this finding. RANO criteria assessment provided a very low rate of pseudoprogression patients due to the stringent nature of these criteria, especially within the first 12 weeks after

chemoradiotherapy, as shown by the low rate of progressive disease during this period. This prevented definitive conclusions regarding the prognostic value of RANO criteria. Some authors reported that while incidence of pseudoprogression strongly depends on the applied criteria, the impact on survival remains the same, regardless of the stringency of the criteria [42]. The prognostic accuracy of different response assessment criteria is, however, yet to be proven in studies of greater magnitude. Nonetheless, the importance of making overcall of early progression difficult with RANO criteria cannot be overemphasized. Failure to exclude patients with pseudoprogression from recurrent gliomas trials with resulting falsely high responses rates and PFS demands the implementation of these strict criteria [11].

The data presented address the issue of the variability of pseudoprogression phenomenon when evaluated with conventional MRI. Novel reliable biochemical and imaging biomarkers are emerging and prospectively designed, and validated investigation is needed to conclude their clinical impact.

5. Conclusions

Pseudoprogression is a phenomenon that appears after chemoradiation therapy for high-grade gliomas, and its pathophysiology is not fully understood. Rates vary widely and are dependent on the method of evaluation. The Macdonald criteria do not take into account all variables implicated in pseudoprogression and are being replaced. In this cohort of GBM patients, the frequency of pseudoprogression varied between 13% and 24%, being over-diagnosed by the older Macdonald criteria, which emphasizes the importance of RANO criteria and new radiological biomarkers for correct response evaluation. The accurate diagnosis of pseudoprogression is essential to avoid modifications of effective therapies or unnecessary treatments for GBM patients. More studies are needed to conclude the prognostic relevance of the different response assessment criteria.

Conflict of Interests

The authors disclose no conflict of interests.

Acknowledgment

The authors acknowledge Francisco Soares, MD, for statistical support.

References

- [1] R. Stupp, W. P. Mason, M. J. Van Den Bent et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *The New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.
- [2] S. Gahramanov, A. M. Raslan, L. L. Muldoon et al., "Potential for differentiation of pseudoprogression from true tumor progression with dynamic susceptibility-weighted contrast-enhanced magnetic resonance imaging using ferumoxytol vs. gadoteridol: a pilot study," *International Journal of Radiation Oncology Biology Physics*, vol. 79, no. 2, pp. 514–523, 2011.
- [3] W. F. Hoffman, V. A. Levin, and C. B. Wilson, "Evaluation of malignant glioma patients during postirradiation period," *Journal of Neurosurgery*, vol. 50, no. 5, pp. 624–628, 1979.
- [4] M. C. Y. de Wit, H. G. de Bruin, W. Eijkenboom, P. A. E. Sillevs Smitt, and M. J. van den Bent, "Immediate post-radiotherapy changes in malignant glioma can mimic tumor progression," *Neurology*, vol. 63, no. 3, pp. 535–537, 2004.
- [5] P. Sanghera, J. Perry, A. Sahgal et al., "Pseudoprogression following chemoradiotherapy for glioblastoma multiforme," *Canadian Journal of Neurological Sciences*, vol. 37, no. 1, pp. 36–42, 2010.
- [6] D. Brandsma and M. J. van den Bent, "Pseudoprogression and pseudoresponse in the treatment of gliomas," *Current Opinion in Neurology*, vol. 22, no. 6, pp. 633–638, 2009.
- [7] R. L. Jensen, "Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudoprogression, and as a therapeutic target," *Journal of Neuro-Oncology*, vol. 92, no. 3, pp. 317–335, 2009.
- [8] G. B. Roldan, J. N. Scott, J. B. McIntyre et al., "Population-based study of pseudoprogression after chemoradiotherapy in GBM," *Canadian Journal of Neurological Sciences*, vol. 36, no. 5, pp. 617–622, 2009.
- [9] L. S. Hu, L. C. Baxter, K. A. Smith et al., "Relative cerebral blood volume values to differentiate high-grade glioma recurrence from posttreatment radiation effect: direct correlation between image-guided tissue histopathology and localized dynamic susceptibility-weighted contrast-enhanced perfusion MR imaging measurements," *American Journal of Neuroradiology*, vol. 30, no. 3, pp. 552–558, 2009.
- [10] C. Tsien, C. J. Galbán, T. L. Chenevert et al., "Parametric response map as an imaging biomarker to distinguish progression from pseudoprogression in high-grade glioma," *Journal of Clinical Oncology*, vol. 28, no. 13, pp. 2293–2299, 2010.
- [11] P. Y. Wen, D. R. Macdonald, D. A. Reardon et al., "Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group," *Journal of Clinical Oncology*, vol. 28, no. 11, pp. 1963–1972, 2010.
- [12] H.-C. Kang, C.-Y. Kim, J. H. Han et al., "Pseudoprogression in patients with malignant gliomas treated with concurrent temozolomide and radiotherapy: potential role of p53," *Journal of Neuro-Oncology*, vol. 102, no. 1, pp. 157–162, 2011.
- [13] A. A. Brandes, E. Franceschi, A. Tosoni et al., "MGMT promoter methylation status can predict the incidence and outcome of pseudoprogression after concomitant radiochemotherapy in newly diagnosed glioblastoma patients," *Journal of Clinical Oncology*, vol. 26, no. 13, pp. 2192–2197, 2008.
- [14] H.-B. Pouleau, N. Sadeghi, D. Balériaux, C. Mélot, O. De Witte, and F. Lefranc, "High levels of cellular proliferation predict pseudoprogression in glioblastoma patients," *International Journal of Oncology*, vol. 40, no. 4, pp. 923–928, 2012.
- [15] H. Motegi, Y. Kamoshima, S. Terasaka et al., "IDH1 mutation as a potential novel biomarker for distinguishing pseudoprogression from true progression in patients with glioblastoma treated with temozolomide and radiotherapy," *Brain Tumor Pathology*, vol. 30, no. 2, pp. 67–72, 2013.
- [16] L. C. Hygino da Cruz Jr., I. Rodriguez, R. C. Domingues, E. L. Gasparetto, and A. G. Sorensen, "Pseudoprogression and pseudoresponse: imaging challenges in the assessment of post-treatment glioma," *American Journal of Neuroradiology*, vol. 32, no. 11, pp. 1978–1985, 2011.
- [17] D.-S. Kong, S. T. Kim, E.-H. Kim et al., "Diagnostic dilemma of pseudoprogression in the treatment of newly diagnosed

- glioblastomas: the role of assessing relative cerebral blood flow volume and oxygen-6-methylguanine-DNA methyltransferase promoter methylation status," *American Journal of Neuroradiology*, vol. 32, no. 2, pp. 382–387, 2011.
- [18] D. R. Macdonald, T. L. Cascino, S. C. Schold Jr., and J. G. Cairncross, "Response criteria for phase II studies of supratentorial malignant glioma," *Journal of Clinical Oncology*, vol. 8, no. 7, pp. 1277–1280, 1990.
 - [19] W. Taal, D. Brandsma, H. G. de Bruin et al., "Incidence of early pseudo-progression in a cohort of malignant glioma patients treated with chemoradiation with temozolomide," *Cancer*, vol. 113, no. 2, pp. 405–410, 2008.
 - [20] M. C. Chamberlain, M. J. Glantz, L. Chalmers, A. van Horn, and A. E. Sloan, "Early necrosis following concurrent Temodar and radiotherapy in patients with glioblastoma," *Journal of Neuro-Oncology*, vol. 82, no. 1, pp. 81–83, 2007.
 - [21] C. Chaskis, B. Neyns, A. Michotte, M. De Ridder, and H. Everaert, "Pseudoprogression after radiotherapy with concurrent temozolomide for high-grade glioma: clinical observations and working recommendations," *Surgical Neurology*, vol. 72, no. 4, pp. 423–428, 2009.
 - [22] J. L. Clarke and S. Chang, "Pseudoprogression and pseudoreponse: challenges in brain tumor imaging," *Current Neurology and Neuroscience Reports*, vol. 9, no. 3, pp. 241–246, 2009.
 - [23] A. Fabi, M. Russillo, G. Metro, A. Vidiri, S. di Giovanni, and F. Cognetti, "Pseudoprogression and MGMT status in glioblastoma patients: implications in clinical practice," *Anticancer Research*, vol. 29, no. 7, pp. 2607–2610, 2009.
 - [24] C. Peca, R. Pacelli, A. Elefante et al., "Early clinical and neuroradiological worsening after radiotherapy and concomitant temozolomide in patients with glioblastoma: tumour progression or radionecrosis?" *Clinical Neurology and Neurosurgery*, vol. 111, no. 4, pp. 331–334, 2009.
 - [25] E. R. Gerstner, M. B. McNamara, A. D. Norden, D. LaFrankie, and P. Y. Wen, "Effect of adding temozolomide to radiation therapy on the incidence of pseudo-progression," *Journal of Neuro-Oncology*, vol. 94, no. 1, pp. 97–101, 2009.
 - [26] E. Yaman, S. Buyukberber, M. Benekli et al., "Radiation induced early necrosis in patients with malignant gliomas receiving temozolomide," *Clinical Neurology and Neurosurgery*, vol. 112, no. 8, pp. 662–667, 2010.
 - [27] A. Gunjur, E. Lau, Y. Taouk, and G. Ryan, "Early post-treatment pseudo-progression amongst glioblastoma multiforme patients treated with radiotherapy and temozolomide: a retrospective analysis," *Journal of Medical Imaging and Radiation Oncology*, vol. 55, no. 6, pp. 603–610, 2011.
 - [28] R. J. Young, A. Gupta, A. D. Shah et al., "Potential utility of conventional MRI signs in diagnosing pseudoprogression in glioblastoma," *Neurology*, vol. 76, no. 22, pp. 1918–1924, 2011.
 - [29] C.-K. Park, J. Kim, S. Y. Yim et al., "Usefulness of MS-MPLA for detection of MGMT promoter methylation in the evaluation of pseudoprogression in glioblastoma patients," *Neuro-Oncology*, vol. 13, no. 2, pp. 195–202, 2011.
 - [30] E. Topkan, S. Topuk, E. Oymak, C. Parlak, and B. Pehlivan, "Pseudoprogression in patients with glioblastoma multiforme after concurrent radiotherapy and temozolomide," *American Journal of Clinical Oncology*, vol. 35, no. 3, pp. 284–289, 2012.
 - [31] D. Brandsma, L. Stalpers, W. Taal, P. Sminia, and M. J. van den Bent, "Clinical features, mechanisms, and management of pseudoprogression in malignant gliomas," *The Lancet Oncology*, vol. 9, no. 5, pp. 453–461, 2008.
 - [32] P. Farace, D. Amelio, G. K. Ricciardi et al., "Early MRI changes in glioblastoma in the period between surgery and adjuvant therapy," *Journal of Neuro-Oncology*, vol. 111, no. 2, pp. 177–185, 2013.
 - [33] C. Meyzer, F. Dhermain, D. Ducreux et al., "A case report of pseudoprogression followed by complete remission after proton-beam irradiation for a low-grade glioma in a teenager: the value of dynamic contrast-enhanced MRI," *Radiation Oncology*, vol. 5, no. 1, article 9, 2010.
 - [34] M. Khasraw and A. B. Lassman, "Advances in the treatment of malignant Gliomas," *Current Oncology Reports*, vol. 12, no. 1, pp. 26–33, 2010.
 - [35] J. G. Pérez-Larraya, M. Lahutte, G. Petrirena et al., "Response assessment in recurrent glioblastoma treated with irinotecan-bevacizumab: comparative analysis of the Macdonald, RECIST, RANO, and RECIST F criteria," *Neuro-Oncology*, vol. 14, no. 5, pp. 667–673, 2012.
 - [36] A. Radbruch, K. Lutz, B. Wiestler et al., "Relevance of T2 signal changes in the assessment of progression of glioblastoma according to the Response Assessment in Neurooncology criteria," *Neuro-Oncology*, vol. 14, no. 2, pp. 222–229, 2012.
 - [37] T. J. Kruser, M. P. Mehta, and H. I. Robins, "Pseudoprogression after glioma therapy: a comprehensive review," *Expert Review of Neurotherapeutics*, vol. 13, no. 4, pp. 389–403, 2013.
 - [38] M. E. Mullins, G. D. Barest, P. W. Schaefer, F. H. Hochberg, R. G. Gonzalez, and M. H. Lev, "Radiation necrosis versus glioma recurrence: conventional MR imaging clues to diagnosis," *American Journal of Neuroradiology*, vol. 26, no. 8, pp. 1967–1972, 2005.
 - [39] Y. Ozsunar, M. E. Mullins, K. Kwong et al., "Glioma recurrence versus radiation necrosis? A pilot comparison of arterial spin-labeled, dynamic susceptibility contrast enhanced MRI, and FDG-PET imaging," *Academic Radiology*, vol. 17, no. 3, pp. 282–290, 2010.
 - [40] P. Weybright, P. C. Sundgren, P. Maly et al., "Differentiation between brain tumor recurrence and radiation injury using MR spectroscopy," *American Journal of Roentgenology*, vol. 185, no. 6, pp. 1471–1476, 2005.
 - [41] Q.-S. Zeng, C.-F. Li, K. Zhang, H. Liu, X.-S. Kang, and J.-H. Zhen, "Multivoxel 3D proton MR spectroscopy in the distinction of recurrent glioma from radiation injury," *Journal of Neuro-Oncology*, vol. 84, no. 1, pp. 63–69, 2007.
 - [42] E. Van Mieghem, A. Wozniak, Y. Geussens et al., "Defining pseudoprogression in glioblastoma multiforme," *European Journal of Neurology*, 2013.

2 GENETIC VARIANTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR PREDICT RISK AND SURVIVAL OF GLIOMAS

Paulo Linhares,^{1,2} Marta Viana-Pereira,^{3,4} Mónica Ferreira,^{3,4} Júlia Amorim,⁵ Rui Nabicxo,⁵ Filipe Pinto,^{3,4} Sandra Costa,^{3,4} Rui Vaz,^{1,2} and Rui Manuel Reis^{3,4,6}

¹Department of Neurosurgery, Hospital S. João, Porto, Portugal; ²Faculty of Medicine, University of Porto, Porto, Portugal; ³Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal; ⁴ICVS/3B's — PT Government Associate Laboratory, Braga/Guimarães, Portugal; ⁵Department of Oncology, Hospital de Braga, Braga, Portugal; ⁶Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, Brazil.



Genetic variants of vascular endothelial growth factor predict risk and survival of gliomas

Tumor Biology
March 2018: 1–11
© The Author(s) 2018
Reprints and permissions:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/1010428318766273
journals.sagepub.com/home/tub

Paulo Linhares^{1,2}, Marta Viana-Pereira^{3,4}, Mónica Ferreira^{3,4},
Júlia Amorim⁵, Rui Naboço⁵, Filipe Pinto^{3,4}, Sandra Costa^{3,4}, Rui Vaz^{1,2} and
Rui Manuel Reis^{3,4,6}

Abstract

The vascular endothelial growth factor regulates angiogenesis that is increased in glioma. VEGF polymorphisms are thought to modulate vascular endothelial growth factor plasma levels and therefore may be implicated in glioma risk. We aimed to clarify the role of VEGF and von Willebrand factor polymorphisms in glioma susceptibility and prognosis. A case-control study of 126 glioma patients and 180 cancer-free controls was performed. Using Sequenom MassARRAY platform, 11 VEGF and 1 VWF polymorphisms were genotyped. Unconditional multivariate logistic regression models were used to calculate odds ratios and 95% confidence intervals. The associations between polymorphisms and survival were evaluated using a Cox regression model. Bonferroni's adjustment was used to correct for multiple testing. The VEGF polymorphism rs833061 was strongly associated with increased risk for glioma (odds ratio = 164.85) and glioblastoma (odds ratio = 155.66), confirmed after Bonferroni correction. Also, the VEGF polymorphisms rs3024994, rs2010963, and particularly the homozygous carriers of rs1005230 were associated with a worse prognosis for glioma and glioblastoma. Our data support a role of VEGF and VWF polymorphisms as glioma biomarkers, with additional potential relevance for molecular stratification of patients for anti-angiogenic therapies.

Keywords

Vascular endothelial growth factor, single-nucleotide polymorphisms, biomarker, risk factor, prognostic factor

Date received: 28 October 2016; accepted: 20 December 2017

Introduction

Gliomas are the most frequent primary malignant central nervous system tumors.^{1–3} These tumors present a multifactorial etiology, being the result of interactions between environmental exposure and genetic susceptibility.⁴ The only proven exogenous environmental cause of glioma is ionizing radiation.^{5,6} Perhaps other environmental causes are involved but remain to be proven. Accordingly, the genetic polymorphisms most frequently studied in the context of brain tumors are located in genes that might influence susceptibility to these tumors in concert with environmental exposures, such as those involved in DNA repair, cell cycle regulation, or immune response.^{7–12} In addition, few genome-wide association studies have focused on gliomas

¹Department of Neurosurgery, Hospital S. João, Porto, Portugal

²Faculty of Medicine, University of Porto, Porto, Portugal

³Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

⁴ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal

⁵Department of Oncology, Hospital de Braga, Braga, Portugal

⁶Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, Brazil

Corresponding author:

Rui Manuel Reis, Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
Email: rreis@med.uminho.pt



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (<http://www.creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).

identifying five risk loci for glioma mostly related to telomerase.^{13–17}

Common pathways in glioma biology include growth factor receptor tyrosine kinases and their signaling pathways. Polymorphisms on the epidermal growth factor (EGF) and its receptor, EGFR, have been identified as risk factors for glioma.^{17–19} In addition, vascular endothelial growth factor (VEGF) is the principal angiogenic factor in both embryonic development and tumor growth and is highly up-regulated in glioblastoma, and its receptors are over-expressed in tumor vessels.²⁰ Importantly, the anti-angiogenic drug bevacizumab, a humanized monoclonal immunoglobulin (Ig) G1 antibody that binds to and inhibits the biological activity of human VEGFA, is currently being explored in different clinical trials involving glioma and has been used in combination with chemotherapy for the treatment of recurrent glioblastoma,^{20–24} which resulted in improved response rates and extended progression-free survival (PFS). Recent studies have shown that some single nucleotide polymorphisms (SNPs) of VEGF/VEGF-related genes could contribute to the modulation of serum VEGF levels, possibly influencing risk and prognosis of solid tumors, including glioma.^{25–27} Moreover, *VEGF* and *VEGFR2* genetic polymorphisms could predict not only patient outcome, as shown by Sjöström et al.,²⁸ but also the response to bevacizumab in glioblastoma patients.²⁹

The von Willebrand factor (VWF) is an essential component of hemostasis at sites of vascular injury.³⁰ The *VWF* gene is highly polymorphic, and SNPs have been found to influence the levels of VWF in the circulation.^{31,32} Moreover, VWF SNPs were also associated with lung cancer risk; however, susceptibility was related with patients' origin.^{33,34}

Given the important role of VEGF signaling in gliomas and the influence tumor risk and prognosis of

particular VEGF/VEGFR polymorphisms in different tumor types, we investigated the relevance of 11 genetic polymorphisms in the 6p12 (VEGFA) and 6p21 (VWF) loci on glioma risk and patient survival.

Materials and methods

Study population

In this case-control study, we enrolled 126 glioma samples from Hospital S. João, Porto, and Hospital de Braga, Braga, Portugal. Tumors were classified according to the World Health Organization (WHO) criteria (Table 1).¹ All patients above 18 years of age, with diagnosis of glioma, treated between 2009 and 2010, and that by informed consent accepted to participate in the study, were included. The patients with different diagnosis or that refused to sign the informed consent were excluded. Complete treatment data were retrieved for 90 patients. Of those, 80 patients underwent surgery, 9 stereotactic biopsy, 77 radiotherapy, 71 chemotherapy (65 underwent Stupp protocol,³⁵ 7 surgery only, 8 surgery + radiotherapy, 5 surgery + radiotherapy + chemotherapy (other than temozolomide), 2 surgery + chemotherapy, and 2 biopsy only), and one additional patient refused treatment. The 180 control samples were selected from cancer-free blood donors at Hospital de Braga, to match for gender and adjust for age in the statistical analysis (Table 1). All patients and controls had a Caucasian background and come from the same region in the north of Portugal. The follow-up information was obtained by consulting clinical records. The control group had no follow-up information. Signed informed consent was obtained from each participant. Local institutional ethics committees of Hospital S. João and Hospital de Braga approved the procedures followed in this study.

Table 1. Clinico-pathological features of gliomas and controls.

Groups (WHO grade)	N	Age, years (median ± SD)	p value ^a	M/F ratio	p value ^a	PFS, months (median)	OS, months (median)
Controls	180	35–85 (53.0 ± 10.8)	p = 0.003	1.69	p = 0.768	n.a.	n.a.
Gliomas (2–4)	126	20–83 (58.0 ± 12.7)		1.63		9 (1–48)	16.5 (1–52)
Astrocytic tumors (2–4)	101	22–83 (59.0 ± 11.9)		1.97		9 (1–40)	16 (1–52)
Diffuse astrocytoma (2)	5	35–67 (41.0 ± 13.5)		0.67		15.5 (9–22)	15.5 (9–22)
Anaplastic astrocytoma (3)	6	32–62 (47.0 ± 12.2)		1.0		36	48
Glioblastoma (4)	86	22–83 (59.5 ± 11.6)		2.07		9 (1–40)	15.5 (1–52)
Gliosarcoma (4)	4	50–72 (61.5 ± 9.1)		All M		5.5 (3–10)	22.5 (7–31)
Oligodendroglioma (2–3)	25	20–78 (50.0 ± 14.1)		0.78		9 (3–48)	23 (6–48)
Oligodendroglioma (2)	7	20–58 (45 ± 13.4)		0.4		48	40.5 (33–48)
Anaplastic oligodendroglioma (3)	18	23–78 (53 ± 14.0)		1.0		8.5 (3–48)	22 (6–48)

WHO: World Health Organization; n.a.: not applicable; SD: standard deviation; M: male; F: female; OS: overall survival; PFS: progression-free survival.

^ap value for independent samples t-test, for testing differences in age and M/F ratio between controls and glioma.

Genotyping

DNA was extracted from the leukocytes of blood samples using the commercially available Citogene® Blood Kit according to the manufacturer's recommendations. Genotyping of the allele-specific primer extension products, which were generated from amplified DNA sequences, was performed using the Sequenom MassARRAY iPLEX Gold platform (Sequenom, San Diego, CA) at the Instituto Gulbenkian de Ciência (IGC), Lisbon, Portugal. Primers were designed using MassARRAY Assay Design 3.1 software (Sequenom), and genotyping was performed in a blinded manner regarding sample status (i.e. case or control subjects). The genotyping quality was assessed by duplicate analysis of 10% of the samples, which demonstrated a 100% agreement rate.

Statistical analysis

Independent samples *t*-test, χ^2 test, and nonparametric Wilcoxon–Mann–Whitney test were used to compare the frequency distribution of age, sex, and genotypes of *VEGF* and *VWF* and the allele distribution among the cases and controls. Moreover, the χ^2 test was used to verify whether the observed allele distribution in the control group was in Hardy–Weinberg equilibrium (HWE). The odds ratio (OR) and 95% confidence intervals (95% CIs) for the effect of the polymorphic variants on the risk for glioma were estimated by multivariate logistic regression analysis, adjusted for gender and age as a continuous variable. Bonferroni's adjustment was used to correct for multiple testing. Associations between polymorphic variants and patient survival were assessed using a multivariate Cox regression model adjusted for patient gender and age. All statistical tests were two-sided, and significance was considered for $p < 0.05$. Data analysis was performed using IBM® SPSS Statistics, version 20.0.

Results

Our cohort of samples included 126 glioma samples (WHO grade II–IV) and 180 cancer-free controls (Table 1). All studied variants were in HWE, apart from the polymorphism rs3025035, for which it was not possible to calculate due to the absence of the homozygous recessive genotype.

Glioma risk assessment

In order to explore the associations between *VEGF* and *VWF* variants and risk for glioma, we used an unconditional multivariate logistic regression analysis adjusted for potential confounding variables (SNP genotypes, patient age, and sex).

We could successfully include on the multivariate logistic analysis 107 cases and 143 controls. The frequencies of each genotype in controls and cases are presented in Table 2 for *VEGF* and *VWF*. The most frequent allele was used to establish the reference genotype. The control group was compared with all glioma cases (WHO grades 2–4), and also with glioblastoma (WHO grade 4), and oligodendroglioma (WHO grades 2–3; Table 2), since these were the most frequent subtypes in our series (Table 1).

We found a significant association between the VEGF SNPs heterozygous rs833061 and risk for glioma and glioblastoma (OR = 164.85, 95% CI = 24.72–1099.54 and OR = 155.66, 95% CI = 17.29–1401.69, respectively, $p < 0.001$ and $p < 0.012$ after Bonferroni correction, Table 2), as well as the variant carriers of this SNP had an increased glioma and glioblastoma risk (OR = 116.54, 95% CI = 20.59–659.69 and OR = 87.73, 95% CI = 12.96–593.67, respectively, $p < 0.001$ and $p < 0.012$ after Bonferroni correction, TT vs TC + CC). Also, the heterozygous rs3024994 was associated with increased risk for glioma (OR = 4.86, 95% CI = 1.29–18.35, $p = 0.020$, Table 2) and glioblastoma (OR = 6.36, 95% CI = 1.31–30.83, $p = 0.022$, Table 2), and the rs25648 variant carriers demonstrated an increased risk for oligodendroglioma (OR = 3.69, 95% CI = 1.11–12.26, $p = 0.033$, Table 2); however, the significance of these results was not confirmed after Bonferroni correction ($p > 0.05$).

Regarding the *VWF* rs9295740 SNP, we observed a significant association between the heterozygous genotype and risk for glioblastoma (OR = 2.24, 95% CI = 1.00–4.98, $p = 0.049$, Table 2); however, the significance of this result was not confirmed after Bonferroni correction ($p > 0.05$).

No statistically significant associations were found between the VEGF SNPs rs3025035, rs3025039, rs3025040, rs1005230, rs699947, rs833070, rs3025010, and rs2010963 variants and risk for glioma, glioblastoma, or oligodendroglioma (Table 2; all p values > 0.05).

Glioma prognostic assessment

In a subset of glioma patients, follow-up data were also available ($n = 57$). The PFS of the samples was 9 months (1–48 months), and the overall survival (OS) was 16.5 months (1–52 months; Table 1). We interrogate the associations between each *VEGF* variant, *VWF*, and PFS or OS by a multivariate Cox proportional hazard model, adjusted for patient age and sex (Table 3). The heterozygous rs3025035 was significantly associated with improved PFS of glioma and glioblastoma (hazard ratio (HR) = 0.15, 95% CI = 0.03–0.73, $p = 0.019$ and HR = 0.11, 95% CI = 0.02–0.72, $p = 0.021$, respectively, Table 3). Improved PFS for

Table 2. Multivariate logistic regression analysis of associations between VEGF/VWF polymorphisms loci and risk for glioma groups.

Polymorphism	Control	Glioma (grades 2–4)	OR (95% CI)	p value ^a	p value ^b	Glioblastoma (grade 4)	OR (95% CI)	p value ^a	p value ^b	Oligodendroglioma (grades 2–3)	OR (95% CI)	p value ^a	p value ^b
<i>rs3025035 (VEGF int7 C/T)</i>													
CC	116	91	–	–	–	63	–	–	–	16	–	–	–
CT	27	16	1.52 (0.61–3.77)	0.369	–	10	1.23 (0.38–3.98)	0.727	–	4	2.04 (0.47–8.89)	0.340	–
TT	0	0	–	–	–	0	–	–	–	0	–	–	–
CT + TT	27	16	1.23 (0.52–2.93)	0.637	–	10	1.08 (0.36–3.24)	0.895	–	4	1.45 (0.36–5.76)	0.599	–
<i>rs3025039 (VEGF + 936 C/T)</i>													
CC	111	82	–	–	–	55	–	–	–	15	–	–	–
CT	29	21	–	–	–	16	–	–	–	4	–	–	–
TT	3	4	2.96 (0.30–29.6)	0.357	–	2	2.73 (0.20–36.87)	0.450	–	1	3.06 (0.16–60.4)	0.462	–
CT + TT	32	25	–	–	–	18	–	–	–	5	–	–	–
<i>rs3025040 (VEGF 3' UTR T/C)</i>													
TT	112	81	–	–	–	54	–	–	–	15	–	–	–
TC	28	22	–	–	–	17	–	–	–	4	–	–	–
CC	3	4	–	–	–	2	–	–	–	1	–	–	–
TC + CC	31	26	–	–	–	19	–	–	–	5	–	–	–
<i>rs1005230 (VEGF –2489 C/T)</i>													
CC	46	29	–	–	–	21	–	–	–	2	–	–	–
CT	73	51	1.09 (0.06–21.44)	0.957	–	34	2.32 (0.10–53.64)	0.600	–	11	–	–	–
TT	24	27	–	–	–	18	–	–	–	7	–	–	–
CT + TT	97	78	0.94 (0.05–16.71)	0.965	–	52	1.80 (0.10–33.75)	0.695	–	18	–	–	–
<i>rs699947 (VEGFA –2578 C/A)</i>													
CC	45	27	–	–	–	19	–	–	–	2	–	–	–
CA	74	53	0.03 (0.001–1.54)	0.082	0.984	36	0.02 (0.00–1.72)	0.087	–	11	–	–	–
AA	24	27	–	–	–	18	–	–	–	7	–	–	–
CA + AA	98	80	0.04 (0.001–1.52)	0.084	–	54	0.03 (0.001–1.36)	0.072	0.864	18	–	–	–
<i>rs833061 (VEGF –460 T/C)</i>													
TT	41	3	–	–	–	3	–	–	–	0	–	–	–
TC	74	77	164.85 (24.72–1099.54)	<0.001	<0.012	52	155.66 (17.29–1401.69)	<0.001	<0.012	13	–	–	–
CC	28	27	–	–	–	18	–	–	–	7	–	–	–
TC + CC	102	104	116.54 (20.59–659.69)	<0.001	<0.012	70	87.73 (12.96–593.67)	<0.001	<0.012	20	–	–	–
<i>rs833070 (VEGF int2 C/A)</i>													
GG	46	28	–	–	–	20	–	–	–	2	–	–	–
GA	73	52	4.01 (0.12–130.16)	0.435	–	35	3.49 (0.08–156.58)	0.520	–	11	–	–	–
AA	24	27	–	–	–	18	–	–	–	7	–	–	–
GA + AA	97	79	3.55 (0.13–97.15)	0.454	–	53	2.82 (0.09–90.97)	0.559	–	18	–	–	–
<i>rs3025010 (VEGF int5 T/C)</i>													
TT	60	42	–	–	–	31	–	–	–	3	–	–	–
TC	72	48	0.57 (0.23–1.41)	0.224	–	31	0.49 (0.17–1.39)	0.181	–	12	0.97 (0.16–5.88)	0.970	–
CC	11	17	1.67 (0.37–7.63)	0.508	–	11	2.97 (0.49–17.88)	0.236	–	5	2.58 (0.18–36.7)	0.484	–
TC + CC	83	65	0.59 (0.25–1.43)	0.243	–	42	0.54 (0.20–1.46)	0.222	–	17	1.23 (0.21–7.35)	0.818	–

(continued)

Table 2. Continued

Polymorphism	Control	Glioma (grades 2–4)	OR (95% CI)	p value ^a	p value ^b	Glioblastoma (grade 4)	OR (95% CI)	p value ^a	p value ^b	Oligodendroglioma (grades 2–3)	OR (95% CI)	p value ^a	p value ^b
<i>rs3024994 (VEGF int2 C/T)</i>													
CC	131	93	–			63	–			18	–		
CT	11	14	4.86 (1.29–18.35)	0.020	0.24	10	6.36 (1.31–30.83)	0.022	0.264	2	1.46 (0.13–16.4)	0.757	–
TT	1	0	–			0	–			0	–		
CT + TT	12	14	2.90 (0.87–9.66)	0.083	0.996	10	3.06 (0.80–11.70)	0.102	–	2	0.98 (0.14–6.98)	0.987	–
<i>rs25648 (VEGF –7 C/T)</i>													
CC	107	73	–			55	–			8	–		
CT	35	31	1.12 (0.52–2.45)	0.770	–	16	0.64 (0.24–1.70)	0.373	–	11	2.82 (0.81–9.81)	0.103	–
TT	1	3	–			2	–			1	–		
CT + TT	36	34	1.63 (0.79–3.37)	0.191	–	18	1.01 (0.42–2.43)	0.987	–	12	3.69 (1.11–12.3)	0.033	0.396
<i>rs2010963 (VEGF –634 G/C)</i>													
GG	58	53	–			36	–			11	–		
GC	71	43	0.96 (0.41–2.25)	0.928	–	28	0.86 (0.31–2.41)	0.771	–	9	1.23 (0.26–5.86)	0.799	–
CC	14	11	1.82 (0.23–14.37)	0.572	–	9	4.09 (0.39–43.41)	0.243	–	0	–		
GC + CC	85	54	0.73 (0.38–1.39)	0.338		37	0.70 (0.33–1.50)	0.357	–	9	0.69 (0.23–2.09)	0.514	–
<i>rs9295740 (VWF G/A)</i>													
GG	94	65	–			41	–			15	–		
GA	40	41	1.62 (0.82–3.22)	0.166	–	31	2.24 (1.00–4.98)	0.049	0.588	5	0.73 (0.18–2.92)	0.652	–
AA	9	1	0.05 (0.002–1.50)	0.084	–	1	0.06 (0.00–2.92)	0.153	–	0	–		
GA + AA	49	42	1.51 (0.79–2.88)	0.213	–	32	1.88 (0.89–3.96)	0.097	–	5	0.92 (0.27–3.22)	0.900	–

VEGF: vascular endothelial growth factor; OR (95% CI): odds ratio with 95% confidence interval.

Bold-faced values indicate significant difference at 5% level.

^ap value without correction.^bp value after Bonferroni correction for multiple comparisons.

Table 3. Multivariate Cox proportional hazard model analysis of associations between VEGF/VWF polymorphisms and glioma patient's survival (adjusted for patient's age and sex).

Polymorphism	Glioma (grades 2–4)				Glioblastoma (grade 4)			
	N	OS HR (95% CI)	p value ^a	p value ^b	N	OS HR (95% CI)	p value ^a	p value ^b
rs325035 (VEGF int7 C/T)								
CC	47	–	–	–	33	–	–	–
CT	10	0.61 (0.17–2.20)	0.448	–	9	0.61 (0.14–2.71)	0.517	–
TT	0	–	–	–	0	–	–	–
CT + TT	10	0.91 (0.30–2.74)	0.862	–	9	0.90 (0.29–2.83)	0.855	–
rs325039 (VEGF + 936 C/T)								
CC	46	–	–	–	33	–	–	–
CT	9	0.61 (0.21–1.78)	0.369	–	8	0.78 (0.24–2.56)	0.682	–
TT	2	0.60 (0.11–3.46)	0.570	–	1	15.94 (0.72–353.16)	0.080	–
CT + TT	11	0.63 (0.29–1.39)	0.257	–	9	0.54 (0.21–1.39)	0.198	–
rs325040 (VEGF 3' UTR T/C)								
TT	46	–	–	–	33	–	–	–
TC	9	–	–	–	8	–	–	–
CC	2	–	–	–	1	–	–	–
TC + CC	11	–	–	–	9	–	–	–
rs1005230 (VEGF –2489 C/T)								
CC	19	–	–	–	14	–	–	–
CT	23	0.22 (0.01–4.78)	0.334	–	16	0.02 (0.00–0.75)	0.035	–
TT	15	85.07 (4.38–1654.35)	0.003	0.036	12	249.48 (8.84–7040.50)	<0.001	<0.012
CT + TT	38	0.78 (0.06–11.06)	0.856	–	28	0.35 (0.02–5.94)	0.464	–
rs699947 (VEGFA –2578 C/A)								
CC	18	–	–	–	13	–	–	–
CA	24	9.77 (0.41–234.92)	0.160	–	17	40.31 (1.07–1515.77)	0.046	0.029
AA	15	–	–	–	12	–	–	–
CA + AA	39	2.80 (0.20–38.55)	0.442	–	29	5.44 (0.32–92.51)	0.241	–
rs833061 (VEGF –460 T/C)								
TT	3	–	–	–	3	–	–	–
TC	39	9.24 (0.88–96.76)	0.063	0.756	27	11.94 (1.04–137.51)	0.047	0.040
CC	15	–	–	–	12	–	–	–
TC + CC	54	12.59 (1.19–38.55)	0.035	0.420	39	16.08 (1.35–191.51)	0.028	0.098
rs833070 (VEGF int2 G/A)								
GG	18	–	–	–	13	–	–	–
GA	24	–	–	–	17	–	–	–
AA	15	–	–	–	12	–	–	–
GA + AA	39	–	–	–	29	–	–	–
rs325010 (VEGF int5 T/C)								
TT	24	–	–	–	18	–	–	–
TC	24	0.48 (0.15–1.60)	0.233	–	17	0.95 (0.26–3.45)	0.936	–
CC	9	0.22 (0.03–1.78)	0.156	–	7	0.04 (0.002–0.63)	0.022	0.060
TC + CC	33	0.53 (0.17–1.64)	0.268	–	24	0.91 (0.26–3.17)	0.880	0.653
					22	0.40 (0.13–1.23)	0.108	–

(continued)

HR (95% CI): hazard ratio with 95% confidence interval; OS: overall survival; PFS: progression-free survival.
 Bold-faced values indicate significant difference at 5% level.
 *p value without correction.
 †p value after Bonferroni correction for multiple comparisons.

by value across dimensions for multiple comparisons

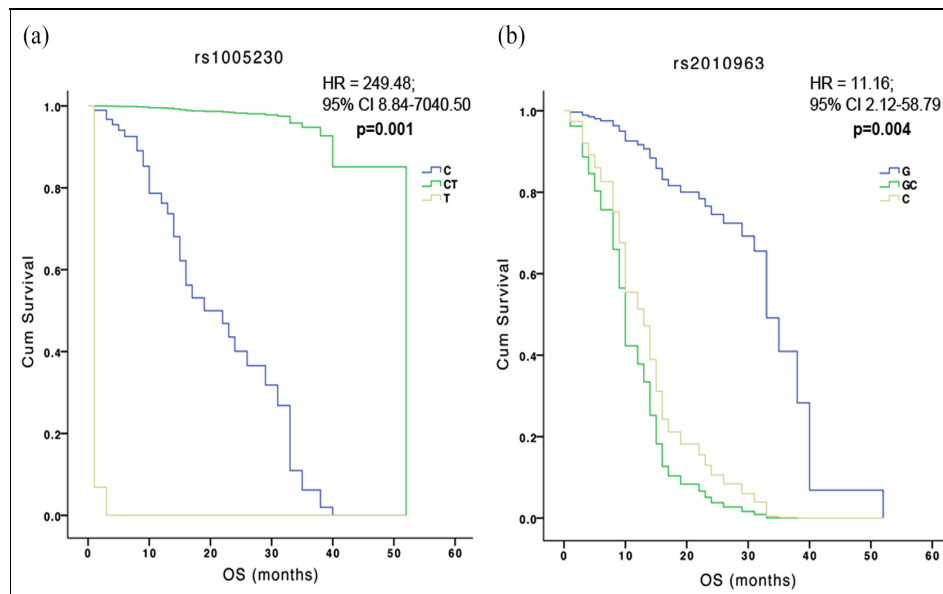


Figure 1. Survival graphs of significant associations between overall survival of glioblastoma patients and VEGF SNPs. Multivariate Cox proportional hazard model regression with respect to glioblastoma patient survival of (a) rs1005230, hazard ratio (HR) = 249.48; 95% confidence interval (CI) = 8.84–7040.50, $p < 0.001$, for TT genotype and (b) rs2010963, HR = 11.16, 95% CI = 2.12–58.79, $p = 0.004$.

glioma was also seen for the heterozygous rs3025010 and for the variant carriers of this SNP (HR = 0.23, 95% CI = 0.08–0.70, $p = 0.009$ and HR = 0.29, 95% CI = 0.11–0.78, $p = 0.015$, respectively, Table 3). On the other hand, glioma patients' carriers of rs833061 variant (HR = 12.59, 95% CI = 1.19–38.55, $p = 0.035$, Table 3) and heterozygous or variant carriers of rs3024994 (HR = 6.52, 95% CI = 1.38–30.82, $p = 0.018$ and HR = 5.08, 95% CI = 1.30–19.81, $p = 0.019$, respectively, for OS and HR = 16.33, 95% CI = 2.79–95.43, $p = 0.002$ and HR = 6.17, 95% CI = 1.51–25.15, $p = 0.011$, respectively, for PFS, Table 3) had a decreased survival. However, after Bonferroni correction, only the heterozygosity of rs3024994 was found to be significantly associated with decreased survival ($p = 0.024$, after correction).

The rs1005230 homozygous carriers presented a decreased OS and PFS both for glioma (OS: HR = 85.07, 95% CI = 4.38–1654.35, $p = 0.003$ and $p = 0.036$ after Bonferroni correction and PFS: HR = 65.72, 95% CI = 3.00–1439.81, $p = 0.008$ and $p = 0.096$ after Bonferroni correction, Table 3) and glioblastoma patients (OS: HR = 249.48, 95% CI = 8.84–7040.50, Figure 1(a) and PFS: HR = 672.46, 95% CI = 12.37–36,562.04, $p < 0.001$ and $p < 0.012$ after Bonferroni correction, Table 3). The heterozygous genotype, has shown to confer an increased OS for glioblastoma patients (HR = 0.02, 95% CI = 0.00–0.75,

$p = 0.035$, Table 3); however, not confirmed after Bonferroni correction.

In addition, when only glioblastoma patients were analyzed, we further observed a decreased survival for rs699947 heterozygous (OS: HR = 40.31, 95% CI = 1.07–1515.77, $p = 0.046$ and PFS: HR = 74.74, 95% CI = 1.55–3594.07, $p = 0.029$, Table 3), rs833061 heterozygous or variant carriers (OS: HR = 11.94, 95% CI = 1.04–137.51, $p = 0.047$ and HR = 16.08, 95% CI = 1.35–191.51, $p = 0.028$, respectively, and PFS: HR = 16.83, 95% CI = 1.13–250.61, $p = 0.040$, Table 3); however, the significance of these results was not confirmed after Bonferroni correction ($p > 0.05$). The rs3024994 heterozygous or variant carriers (OS: HR = 7.89, 95% CI = 1.22–51.19, $p = 0.03$ and HR = 6.05, 95% CI = 1.08–33.94, $p = 0.041$, respectively, and PFS: HR = 176.32, 95% CI = 8.58–3622.74, $p < 0.001$ and $p < 0.012$ after Bonferroni correction, and HR = 16.84, 95% CI = 2.60–109.26, $p = 0.003$ and $p = 0.036$ after Bonferroni correction, Table 3) and rs2010963 heterozygous and variant homozygous (OS: HR = 11.16, 95% CI = 2.12–58.79, $p = 0.004$ and $p = 0.048$ after Bonferroni correction, Figure 1(b) and PFS: HR = 20.10, 95% CI = 1.45–279.11, $p = 0.025$ and $p = 0.300$ after Bonferroni correction, respectively, Table 3). Finally, rs3025010 variant homozygous carriers had an improved OS for glioblastoma (HR = 0.04, 95% CI = 0.002–0.63,

$p = 0.022$ and $p = 0.264$ after Bonferroni correction, Table 3).

Discussion

VEGF is the major proangiogenic factor involved in tumor growth and progression, including glioblastoma, being over-expressed in tumor vessels.²⁰ VEGF–VEGFR signaling pathway stimulates not only angiogenic signals but also the secretion of the VWF from endothelial cells, and VWF was described as having a potential role on angiogenesis.^{36–38} We have studied the contribution of 11 *VEGF* and 1 *VWF* polymorphisms to the risk and outcome of glioma and glioblastoma in the Portuguese population. These polymorphisms were selected based on some of the several studies that reported that some SNPs in the *VEGF* and *VWF* loci may conditionate serum VEGF expression modulation and therefore confer tumor risk.^{25–27,34,39} These polymorphisms were reported to modify VEGFA serum levels, in particular rs3025039 and rs2010963, and to modulate risk, aggressiveness prognosis of different solid tumors, specifically rs3025035, rs3025040, rs699947, rs833061, rs833070, rs3025010, and rs3024994.^{34,40–44} Moreover, anti-angiogenic therapies are currently in clinical use for glioma patients, and allelic variations of *VEGF* and *VEGFR* genes have been associated with glioma patient responses to those therapies.²⁸

We found that, in our population, the *VEGF* rs833061 SNP conferred an increased risk for glioma and glioblastoma. Strikingly, rs833061 T/C heterozygotes were over 150 times as likely to develop glioblastoma (OR = 155.66, 95% CI = 17.29–1401.69) or glioma (WHO grade 2–4; OR = 164.85, 95% CI = 24.72–1099.54). Although the physiological or pathological function of the SNP rs833061 in glioblastoma is still unknown, this SNP has already shown to be related to increased PFS in response to bevacizumab in combination with sorafenib in recurrent glioblastoma patients.²⁹

The rs3024994 C/T genotype presented an OR = 4.86 (95%CI = 1.29–18.35) for glioma and OR = 6.36 (95% CI = 1.31–30.83) for glioblastoma and also the *VWF* rs9295749 SNP was associated with an increased risk for glioblastoma; however, the significance of this result was not confirmed after Bonferroni correction ($p > 0.05$). Li et al.²⁷ have reported that rs3024994 variant was associated with a decreased risk, whereas rs2010963 variant carriers and rs3025039 variant homozygous had an increased risk for glioma. Also Jiang et al.⁴⁰ described rs3025039 and rs2010963 variant homozygosity to be associated with an increased risk for glioma. These discrepancies could be related to different background of the populations

studied. While the previous works have studied Chinese patients, our samples are from Caucasian origin. Although no significant differences in genotype or allele frequencies are observed between different population origins,⁴⁵ it was already reported for other diseases that these polymorphisms differentially related to risk in distinctive populations.^{46,47}

Importantly, *VEGF* polymorphisms have been related with patients' response to anti-angiogenic drugs, particularly bevacizumab.^{29,48} Few studies have described an association between *VEGF* SNPs, specifically rs2010963, rs699947, rs833061, and rs1005230 and response to bevacizumab in glioblastoma patients. In particular, the rs2010963, rs699947, and rs833061 variant homozygous genotypes were associated with an increase in PFS, whereas the rs1005230 variant homozygous genotype was associated with a decrease in PFS of glioblastoma patients following bevacizumab treatment. A major limitation of our study is the small number of patients treated with bevacizumab ($n = 8$), which did not allow any statistical analysis to access the value of the studied polymorphisms in the context of the bevacizumab treatment. Therefore, we have also analyzed the effect of these polymorphisms on patients' survival, independently of the treatment regimen applied.

The rs1005230 variant homozygous consistently presented a decreased OS and PFS both for glioma and glioblastoma patients. Similarly, the rs3024994 variant heterozygous consistently presented a decreased PFS both for glioma and glioblastoma patients. Also the rs2010963 heterozygous glioblastoma patients had a reduced OS.

In conclusion, our data strongly suggest that different *VEGF* SNPs highly increase the risk to develop gliomas, and specifically glioblastoma, particularly the presence of the rs833061 variant. Moreover, several *VEGF* SNPs seem to be prognostic markers of glioma and glioblastoma patients' survival. In particular, rs3024994 heterozygous and the homozygous carriers of rs1005230 confer a worse prognosis for these patients. Although these data need confirmation by independent studies, they demonstrate that *VEGF* SNPs are potential glioblastoma biomarkers.

Acknowledgements

Paulo Linhares and Marta Viana-Pereira contributed equally to this work.

Compliance with ethical standards

This study was approved by “Comissão de Ética para a Saúde,” stating that it was unanimously approved, with nothing to object. Signed informed consent was obtained from each participant. Local institutional ethics committees of Hospital São João, Portugal and Hospital de Braga, Portugal, approved the procedures followed in this study.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was funded by Fundação para a Ciência e Tecnologia (FCT), Portugal (www.fct.pt; grant PTDC/SAU-ONC/115513/2009_FCOMP-01-0124-FEDER-015949 to R.M.R.). This article has been developed under the scope of the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER). M.V.-P. is recipient of an FCT Post-Doctoral fellowship (ref: SFRH/BPD/104290/2014).

References

- Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 2007; 114(2): 97–109.
- Tanaka S, Louis DN, Curry WT, et al. Diagnostic and therapeutic avenues for glioblastoma: no longer a dead end? *Nat Rev Clin Oncol* 2013; 10(1): 14–26.
- Weller M, van den Bent M, Hopkins K, et al. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *Lancet Oncol* 2014; 15(9): e395–e403.
- Ostrom QT, Gittleman H, Stetson L, et al. Epidemiology of gliomas. *Cancer Treat Res* 2015; 163: 1–14.
- Ohgaki H. Epidemiology of brain tumors. *Methods Mol Biol* 2009; 472: 323–342.
- Wrensch M, Fisher JL, Schwartzbaum JA, et al. The molecular epidemiology of gliomas in adults. *Neurosurg Focus* 2005; 19(5): E5.
- Adel Fahmideh M, Schwartzbaum J, Frumento P, et al. Association between DNA repair gene polymorphisms and risk of glioma: a systematic review and meta-analysis. *Neuro Oncol* 2014; 16(6): 807–814.
- Liu Y, Scheurer ME, El-Zein R, et al. Association and interactions between DNA repair gene polymorphisms and adult glioma. *Cancer Epidemiol Biomarkers Prev* 2009; 18(1): 204–214.
- McKean-Cowdin R, Barnholtz-Sloan J, Inskip PD, et al. Associations between polymorphisms in DNA repair genes and glioblastoma. *Cancer Epidemiol Biomarkers Prev* 2009; 18(4): 1118–1126.
- Zawlik I, Kita D, Vaccarella S, et al. Common polymorphisms in the MDM2 and TP53 genes and the relationship between TP53 mutations and patient outcomes in glioblastomas. *Brain Pathol* 2009; 19(2): 188–194.
- Pandey JP, Kaur N, Costa S, et al. Immunoglobulin genes implicated in glioma risk. *Oncoimmunology* 2014; 3: e28609.
- Vieira de Castro J, Goncalves CS, Costa S, et al. Impact of TGF- β 1 -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis. *Tumour Biol* 2015; 36(8): 6525–6532.
- Shete S, Hosking FJ, Robertson LB, et al. Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet* 2009; 41(8): 899–904.
- Walsh KM, Codd V, Smirnov IV, et al. Variants near TERT and TERC influencing telomere length are associated with high-grade glioma risk. *Nat Genet* 2014; 46(7): 731–735.
- Walsh KM, Rice T, Decker PA, et al. Genetic variants in telomerase-related genes are associated with an older age at diagnosis in glioma patients: evidence for distinct pathways of gliomagenesis. *Neuro Oncol* 2013; 15(8): 1041–1047.
- Yang TH, Kon M, Hung JH, et al. Combinations of newly confirmed glioma-associated loci link regions on chromosomes 1 and 9 to increased disease risk. *BMC Med Genomics* 2011; 4: 63.
- Rajaraman P, Melin BS, Wang Z, et al. Genome-wide association study of glioma and meta-analysis. *Hum Genet* 2012; 131(12): 1877–1888.
- Costa BM, Ferreira P, Costa S, et al. Association between functional EGF + 61 polymorphism and glioma risk. *Clin Cancer Res* 2007; 13(9): 2621–2626.
- Costa BM, Viana-Pereira M, Fernandes R, et al. Impact of EGFR genetic variants on glioma risk and patient outcome. *Cancer Epidemiol Biomarkers Prev* 2011; 20(12): 2610–2617.
- Plate KH, Scholz A and Dumont DJ. Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. *Acta Neuropathol* 2012; 124(6): 763–775.
- Curry RC, Dahiya S, Alva Venur V, et al. Bevacizumab in high-grade gliomas: past, present, and future. *Expert Rev Anticancer Ther* 2015; 15(4): 387–397.
- Ghiaseddin A and Peters KB. Use of bevacizumab in recurrent glioblastoma. *CNS Oncol* 2015; 4(3): 157–69.
- Mesti T, Moltara ME, Boc M, et al. Bevacizumab and irinotecan in recurrent malignant glioma, a single institution experience. *Radiol Oncol* 2015; 49(1): 80–85.
- Carvalho BFA, Almeida D, Sampaio L, et al. Second-line chemotherapy in recurrent glioblastoma: a 2-cohort study. *Oncol Res Treat* 2015; 38(7–8): 348–354.
- Jain L, Vargo CA, Danesi R, et al. The role of vascular endothelial growth factor SNPs as predictive and prognostic markers for major solid tumors. *Mol Cancer Ther* 2009; 8(9): 2496–2508.
- Watson CJ, Webb NJ, Bottomley MJ, et al. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine* 2000; 12(8): 1232–1235.
- Li R, Zhao Y, Fan W, et al. Possible association between polymorphisms of human vascular endothelial growth factor A gene and susceptibility to glioma in a Chinese population. *Int J Cancer* 2011; 128(1): 166–175.
- Sjostrom S, Wibom C, Andersson U, et al. Genetic variations in VEGF and VEGFR2 and glioblastoma outcome. *J Neurooncol* 2011; 104(2): 523–527.
- Galanis E, Anderson SK, Lafky JM, et al. Phase II study of bevacizumab in combination with sorafenib in recurrent glioblastoma (N0776): a north central cancer

- treatment group trial. *Clin Cancer Res* 2013; 19(17): 4816–4823.
30. James PD and Goodeve AC. von Willebrand disease. *Genet Med* 2011; 13(5): 365–376.
 31. Mancuso DJ, Tuley EA, Westfield LA, et al. Structure of the gene for human von Willebrand factor. *J Biol Chem* 1989; 264(33): 19514–19527.
 32. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 1998; 67: 395–424.
 33. TanTai J, Shen Y and Zhao H. Quantitative assessment of the influence of common variations on 6p21 and lung cancer risk. *Tumour Biol* 2014; 35(1): 689–694.
 34. De Mello RA, Ferreira M, Soares-Pires F, et al. The impact of polymorphic variations in the 5p15, 6p12, 6p21 and 15q25 Loci on the risk and prognosis of Portuguese patients with non-small cell lung cancer. *PLoS ONE* 2013; 8(9): e72373.
 35. Hegi ME, Diserens AC, Godard S, et al. Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. *Clin Cancer Res* 2004; 10(6): 1871–1874.
 36. Xiong Y, Huo Y, Chen C, et al. Vascular endothelial growth factor (VEGF) receptor-2 tyrosine 1175 signaling controls VEGF-induced von Willebrand factor release from endothelial cells via phospholipase C-gamma 1- and protein kinase A-dependent pathways. *J Biol Chem* 2009; 284(35): 23217–23224.
 37. Yang X, Sun HJ, Li ZR, et al. Gastric cancer-associated enhancement of von Willebrand factor is regulated by vascular endothelial growth factor and related to disease severity. *BMC Cancer* 2015; 15: 80.
 38. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood* 2011; 117(3): 1071–1080.
 39. Zhao P, Chen A, Qi Q, et al. Impact of VEGFA polymorphisms on glioma risk in Chinese. *Oncotarget* 2017; 8(48): 83712–83722.
 40. Jiang H, Lian M, Xie J, et al. Three single nucleotide polymorphisms of the vascular endothelial growth factor (VEGF) gene and glioma risk in a Chinese population. *J Int Med Res* 2013; 41(5): 1484–1494.
 41. Jeon YJ, Kim JW, Park HM, et al. Interplay between 3'-UTR polymorphisms in the vascular endothelial growth factor (VEGF) gene and metabolic syndrome in determining the risk of colorectal cancer in Koreans. *BMC Cancer* 2014; 14: 881.
 42. Tu J, Wang S, Zhao J, et al. rs833061 and rs699947 on promoter gene of vascular endothelial growth factor (VEGF) and associated lung cancer susceptibility and survival: a meta-analysis. *Med Sci Monit* 2014; 20: 2520–2526.
 43. Beeghly-Fadiel A, Shu XO, Lu W, et al. Genetic variation in VEGF family genes and breast cancer risk: a report from the Shanghai Breast Cancer Genetics Study. *Cancer Epidemiol Biomarkers Prev* 2011; 20(1): 33–41.
 44. Ruggiero D, Dalmaso C, Nutile T, et al. Genetics of VEGF serum variation in human isolated populations of Cilento: importance of VEGF polymorphisms. *PLoS ONE* 2011; 6(2): e16982.
 45. Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature* 2012; 491(7422): 56–65.
 46. Lu Y, Ge Y, Shi Y, et al. Two polymorphisms (rs699947, rs2010963) in the VEGFA gene and diabetic retinopathy: an updated meta-analysis. *BMC Ophthalmol* 2013; 13: 56.
 47. Xu X, Du C, Li H, et al. Association of VEGF genetic polymorphisms with recurrent spontaneous abortion risk: a systematic review and meta-analysis. *PLoS ONE* 2015; 10(4): e0123696.
 48. DiStefano AL, Labussiere M, Lombardi G, et al. VEGFA SNP rs2010963 is associated with vascular toxicity in recurrent glioblastomas and longer response to bevacizumab. *J Neurooncol* 2015; 121(3): 499–504.

3 THE IMPORTANCE OF THE NEUTROPHIL - TO - LYMPHOCYTE RATIO IN THE PROGNOSIS OF GLIOMA AND ITS SUBTYPES

Paulo Linhares,^{1,2,3} Axel Ferreira⁴ Rui Vaz,^{1,2,3}

¹Serviço de Neurocirurgia do Centro Hospitalar São João, Porto, Portugal; ²Faculdade de Medicina da Universidade do Porto, Porto, Portugal; ³Unidade de Neurociências do Hospital CUF Porto, Porto, Portugal;

⁴Serviço de Neurologia do Hospital Pedro Hispano, Matosinhos, Portugal.

The importance of the neutrophil-to-lymphocyte ratio in the prognosis of glioma and its subtypes

Dear editor

We read with great interest the original article by Wang Z. et al that found a correlation between neutrophil-to-lymphocyte ratio (NLR) and glioma prognosis. His group detected, in their patient cohort, a correlation between NLR <3.2 and better prognosis and also between NLR >3.2 and poor prognosis.¹ The subdivision of these tumors between neutrophil type and lymphocyte type could contribute to a better stratification of patient prognosis with a simple and relatively innocuous blood test.

In fact, it is known for many years that inflammation promotes angiogenesis and proliferation of malignant cells.² In that matter, knowing that NLR is a marker of inflammation, it is logical that NLR can be an independent risk factor for worse prognosis in several malignancies.^{3,4} And, as it is acknowledged by the mentioned paper, similar findings have been made before in gliomas.^{5,6} Nonetheless, relating NLR values with WHO grades of malignancy can be a limitative way of using this potential new tool. It is already established that different types of glioma have different prognosis, and the endeavor of the WHO classification, especially the 2016 update, is exactly that, trying to classify tumors in subtypes and grade them according to prognosis.⁷ In that respect, we think that the use of NLR should not be restrained to predict the grade of a glial tumor.

Following this train of thought, we feel that the discussion should be centered in the effect of NLR values in the prognosis of the different glioma subtypes, specifically the ones that are more common and aggressive. Much like the discovery of the relation between IDH1 mutations and better prognosis in glioblastoma (GBM),⁸ we think that the NLR can have an analogous role in certain glioma subtypes. Interestingly, recent research by Auezova et al have shown that IDH1 mutations in glioma are correlated to lower NLR values. As NLR is a marker for chronic inflammation, they infer that IDH1 mutations are related to lower levels of chronic inflammation, and therefore to a better prognosis.⁹ In retrospective, we think that the main constraint of the Wang Z. et al paper is the fact that all grades of glioma were considered and, for us, the question is not what is the behavior of NLR among the grades, but what we can achieve among the same grade.

In previous work made by our group and published in the Journal of Neuro-oncology, we analyzed a cohort of 140 patients with GBM submitted to resection surgery between January 2005 and January

2013 in a tertiary care hospital. As adjunctive treatment, 117 patients completed the Stupp protocol. As for the remaining patients, 14 patients did no adjunctive therapy and 9 underwent only standard or hypofractionated radiotherapy, or only chemotherapy with Temozolomide or Carmustine due to poor functional status. Mean age at surgery was 62.9 ± 10.0 years, and mean age at death was 64.4 ± 9.8 years. Mean overall survival was 19.4 ± 14.3 months, and mean progression-free survival was 9.4 ± 8.7 months. In the complete cohort, we did not find a correlation between NLR and overall survival; however, in the subgroup of 117 patients that completed Stupp protocol (radiotherapy and chemotherapy with Temozolomide), we found that a preoperative NLR >7 correlated with a shorter overall survival (Figure 1).¹⁰ Other groups have found similar results.¹¹⁻¹⁴ Most significantly Bambury et al found, in a cohort of 84 GBM patients, that preoperative NLR >4 was associated with worse median overall survival.¹¹ And McNamara et al published

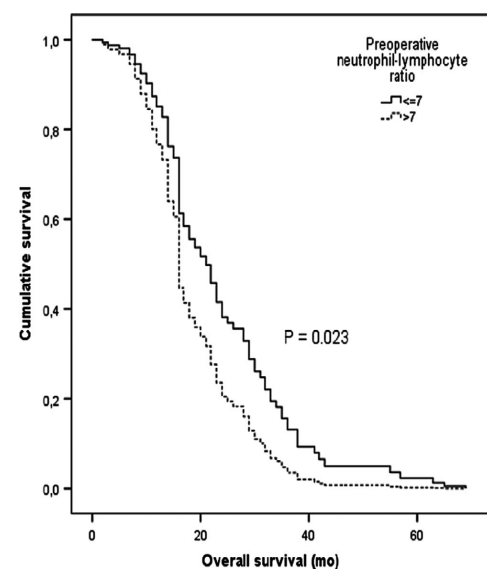


FIGURE 1 Kaplan-Meier plot of overall survival stratified by neutrophil-lymphocyte ratio value (cut-off = 7.0)—Stupp protocol treated patients

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2019 The Authors. CNS Neuroscience & Therapeutics Published by John Wiley & Sons Ltd.

identical conclusions in a cohort of 107 GBM patients undergoing second surgery.¹² All the prementioned studies had their limitations, in the sense that they were all retrospective and therefore prone to bias. In that matter, we feel that prospective studies should be done to confirm this finding. However, we feel that the main goal should be guided toward studying specific subtypes, one because different types of glioma have different known prognosis, which renders an holistic sample of little utility, and two because many of the observed cases will be of GBM, which has an already known relation between NLR and prognosis, and can contaminate the sample.

Interestingly, our research also found that the relation with prognosis was only significant in the patients that followed optimal therapy. This can suggest that parameters like NLR may, in the future, modulate therapy options, because it implies that a more aggressive approach can be futile in more aggressive types of GBM.

As it has been said, we think that the use of NLR shouldn't be limited to predicting the grade of malignancy in glial tumors. In the greater picture, it can be considered as a simple easy access tool to better establish prognosis in certain glioma subtypes, as it appears to be the case in GBM, and really change, in a daily basis, our therapeutic approach.

CONFLICT OF INTEREST

The authors have no disclosures or conflict of interest to declare.

Paulo Linhares^{1,2,3}

Axel Ferreira⁴ 

Rui Vaz^{1,2,3}

¹Serviço de Neurocirurgia do Centro Hospitalar São João, Porto, Portugal

²Faculdade de Medicina da Universidade do Porto, Porto, Portugal

³Unidade de Neurociências do Hospital CUF Porto, Porto, Portugal

⁴Serviço de Neurologia do Hospital Pedro Hispano, Matosinhos, Portugal

Correspondence

Axel Ferreira, Serviço de Neurologia, Hospital Pedro

Hispano, R. de Dr. Eduardo Torres, Sra. da Hora, Matosinhos, 4464-513, Portugal.
Email: axelferr@gmail.com

ORCID

Axel Ferreira  <https://orcid.org/0000-0003-3792-8542>

REFERENCES

- Wang Z-L, Zhang C-B, Liu Y-Q, Wang Z, Jiang T. Peripheral blood test provides a practical method for glioma evaluation and prognosis prediction. *CNS Neurosci Ther*. 2019;25(8):876-883.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
- Templeton AJ, McNamara MG, Seruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2014;106(6):dju124.
- Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol*. 2013;88:218-230.
- Auezova R, Ryskeldiev N, Doskaliyev A, et al. Association of preoperative levels of selected blood inflammatory markers with prognosis in gliomas. *Onco Targets Ther*. 2016;9:6111-6117.
- Zhang J, Zhang S, Song Y, et al. Prognostic role of neutrophil lymphocyte ratio in patients with glioma. *Oncotarget*. 2017;8(35):59217-59224.
- Wesseling P, Capper D. WHO 2016 classification of gliomas. *Neuropathol Appl Neurobiol*. 2018;44(2):139-150.
- Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807-1812.
- Auezova R, Ivanova N, Akshulakov S, et al. Isocitrate dehydrogenase 1 mutation is associated with reduced levels of inflammation in glioma patients. *Cancer Manag Res*. 2019;15(11):3227-3236.
- Lopes M, Carvalho B, Vaz R, Linhares P. Influence of neutrophil-lymphocyte ratio in prognosis of glioblastoma multiforme. *J Neurooncol*. 2018;136(1):173-180.
- Bambury RM, Teo MY, Power DG, et al. The association of pre-treatment neutrophil to lymphocyte ratio with overall survival in patients with glioblastoma multiforme. *J Neurooncol*. 2013;114:149-154.
- McNamara MG, Lwin Z, Jiang H, et al. Factors impacting survival following second surgery in patients with glioblastoma in the temozolomide treatment era, incorporating neutrophil/lymphocyte ratio and time to first progression. *J Neurooncol*. 2014;117:147-152.
- Han S, Liu Y, Li Q, Li Z, Hou H, Wu A. Pre-treatment neutrophil-to-lymphocyte ratio is associated with neutrophil and T-cell infiltration and predicts clinical outcome in patients with glioblastoma. *BMC Cancer*. 2015;15:617-626.
- Wang PF, Song HW, Cai HQ, et al. Preoperative inflammation markers and IDH mutation status predict glioblastoma patient survival. *Oncotarget*. 2017;8(30):50117-50123.

4 SERUM LACTATE LEVELS ARE ASSOCIATED WITH GLIOMA MALIGNANCY GRADE

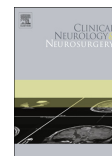
Mariana Branco,^{a,b,*,1} Paulo Linhares,^{b,c,1} Bruno Carvalho,^c Patrícia Santos,^d Bruno M. Costa,^e Rui Vaz,^{b,c}

^a Serviço de Neurologia, Centro Hospitalar de Entre Douro e Vouga, Portugal; ^b Faculdade de Medicina da Universidade do Porto, Portugal; ^c Serviço de Neurocirurgia, Centro Hospitalar de São João, Portugal; ^d Serviço de Anestesiologia, Centro Hospitalar de São João, Portugal; ^e Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Portugal.



Contents lists available at ScienceDirect

Clinical Neurology and Neurosurgery

journal homepage: www.elsevier.com/locate/clineuro

Serum lactate levels are associated with glioma malignancy grade

Mariana Branco^{a,b,*}, Paulo Linhares^{b,c,1}, Bruno Carvalho^c, Patrícia Santos^d, Bruno M. Costa^e, Rui Vaz^{b,c}^a Serviço de Neurologia, Centro Hospitalar de Entre Douro e Vouga, Portugal^b Faculdade de Medicina da Universidade do Porto, Portugal^c Serviço de Neurocirurgia, Centro Hospitalar de São João, Portugal^d Serviço de Anestesiologia, Centro Hospitalar de São João, Portugal^e Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Portugal

ARTICLE INFO

Keywords:

Glioma

Lactate

Biomarker

Diagnosis

Neuro-oncology

Neurosurgery

ABSTRACT

Objective: Recent studies have suggested that high grade gliomas are associated with elevated serum lactate concentrations. The aim of the present study is to assess these findings in a sample of patients.**Patients and methods:** We reviewed the anesthetic charts of patients with low-grade and high-grade glioma who underwent resection surgery and collected serum lactate concentration before tumor resection, as well as other demographic and tumor-related data (age, gender, WHO grade, and size of the tumor). A statistical comparison between patients with normal (< 2 mmol/L) and elevated (≥ 2 mmol/L) serum lactate was performed.**Results:** We included a total of 152 patients (mean age 49.07 years). 62.5% of patients ($n = 95$) had a high-grade glioma and 37.5% ($n = 67$) a low-grade glioma. The multivariate regression showed that high grade gliomas had significantly higher lactate concentration ($p < 0.01$). The OR for elevated pre-resection serum lactate increased from 4.94 to 14.33 after adjusting for age and pre-surgical corticosteroid use, and the AUC for the final regression model was 0.98.**Conclusion:** This study reinforces the role of serum lactate as a potential biomarker of brain tumors malignancy, and its results encourage further research on this subject, in order to improve the understanding of this phenomenon and to assess its potential as prognostic and therapeutic monitoring tool.

1. Introduction

Malignant gliomas are the most common type of primary malignant brain tumors [1]. Glioblastoma (GBM) is a high grade glioma (WHO grade IV) and the most common primary central nervous system (CNS) tumor in European countries and USA [2]. The etiology of GBM is still under debate, but both genetic and epigenetic factors are involved, and glioma stem cells have a major role. The 2016 edition of the World Health Organization (WHO) classification of tumors of the CNS [3] was the first to include guidelines for the use of molecular markers of glioma, indicating that genetic molecular diagnosis and gene analysis of the disease play an increasingly important role [4].

Molecular biomarkers could be an important tool in distinguishing different subtypes of tumors. Therefore, there is a growing need to identify biomarkers of GBM that may have clinical value [5]. The emergence of molecular biomarkers had a significant impact on histological typing and diagnosis, as well as predicting patient survival and

response to treatment [6]. However, those biomarkers typically require complex laboratory analyses and are not suitable for pre-operative diagnosis.

Despite the significant progress made in the understanding of oncogenetics and molecular biology of the tumor in recent years, the discovery of therapeutic approaches with significant clinical benefit for GBM patients remains a challenge [4]. In addition to genetic biomarkers, the value of various metabolites as cancer biomarkers have been widely investigated.

Lactate is a final metabolic product of highly proliferative tumors, like GBM, and results from a mechanism of survival of the tumor, which effluxes lactic acid to its microenvironment through transmembrane monocarboxylate transporters (MCTs). According to the conclusions of an *in vivo* study, inhibition of MCTs function markedly impaired glioma invasion [7]. It is known that hyperlactacidemia may occur in cases of malignancy, specially hematological cancers with liver involvement [8]. We have noticed that a significant number of patients with brain

* Corresponding author at: Neurology Department, Centro Hospitalar de Entre o Douro e Vouga, R. Dr. Cândido Pinho 5, 4520-211, Santa Maria da Feira, Portugal.
E-mail address: mariana_c_branco@hotmail.com (M. Branco).

¹ These authors contributed equally to this work.

<https://doi.org/10.1016/j.clineuro.2019.105546>

Received 26 May 2019; Received in revised form 29 July 2019; Accepted 30 September 2019

Available online 01 October 2019

0303-8467/ © 2019 Elsevier B.V. All rights reserved.

tumors undergoing surgical gross resection or biopsy presented high serum lactate levels (≥ 2 mmol/L) before or at the beginning of the surgery, without an evident explaining factor. In the majority of cases, the histopathological examination revealed high grade glioma. There is some data supporting a correlation between tumor grade and high pre-surgical serum lactate level [9,10], therefore we aimed to validate this association in our cohort of patients.

2. Materials and methods

2.1. Study population

We reviewed the clinical files and anesthetic charts of 152 patients who underwent brain tumor surgery at Hospital São João, a European Union tertiary hospital, from January 2006 to April 2016. We included patients with low-grade and high-grade glioma, who had records of serum lactate concentration obtained by arterial blood gases performed in the operating room after induction of general anesthesia and before skin incision. Additional blood gases were collected during tumor resection but were used only for patient monitoring and were not used for statistical analysis. Patients with known liver or renal dysfunction and with congestive heart failure were excluded, as well as patients who had hemodynamically unstable periods previously to blood gases collection, with necessity of inotropic drugs or blood transfusion ($n = 4$). In addition to serum lactate concentration, we collected demographic, tumor-related data (age, gender, WHO grade, and size of the tumor) and reviewed the clinical files to assess pre-surgery corticosteroid use (in the previous 72 h). A pre-resection lactate concentration ≥ 2 mmol/L was considered as "elevated", according to several studies in literature [11–13]. For tumor volume we performed a diameter-based measurement using MRI imaging and divided them into larger or smaller than 50 mm.

2.2. Statistical analysis

Comparisons between patients with normal (< 2 mmol/L) and elevated (≥ 2 mmol/L) lactate were performed using the Student's *t*-test for independent samples or χ^2 test, where appropriate. The tests were two-sided, with a significance level of 0.05. To measure associations between tumor grade (high and low grade), serum lactate concentration, and the different clinical and demographic variables we calculated crude and adjusted odds ratio (OR), using univariable and multivariable logistic regression. We fitted all the variables that were significant predictors of tumor grade in a logistic regression model. We assessed the predictive value of an elevated serum concentration to determine tumor grade by estimating the area under the curve of the final model. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 23.

3. Results

We included a total of 152 patients, 88 males and 64 females. The mean age was 49.07 (standard deviation(SD) = 19.72 years), 62.5% of patients ($n = 95$) had a high-grade glioma and 37.5% ($n = 67$) a low-grade (Table 1). Patients with high-grade glioma were on average older than patients with low-grade glioma (58.6 vs. 33.12 years old; $p < 0.01$). In low-grade gliomas, the mean lactate concentration was 1.38 mmol/L (SD = 0.61), and in high-grade gliomas it was 2.03 (SD = 0.81).

There were 42.1% patients with elevated pre-resection serum lactate ($n = 64$), and the mean lactate level was 1.79 mmol/L. When comparing patients divided according to their serum lactate (normal (< 2 mmol/L) versus elevated (≥ 2 mmol/L)) we found significant associations between higher serum lactate and higher tumor grade and female gender ($p < 0.01$), and no significant associations with age or tumor size (Table 2). There was a significant association between pre-

Table 1
Demographics and clinical characteristics of the study patients.

Gender, %	
Male	57.9%
Female	42.1%
Age, years (mean, SD)	49.07 (19.72)
Tumor grade, %	
High	62.5%
Low	37.5%
Tumor size	
≥ 50 mm	34.9%
< 50 mm	48.0%
Serum lactate, %	
Elevated	42.1%
Normal	57.9%
Serum lactate concentration, mmol/L (mean, SD)	1.79 (0.81)

Table 2
Comparison of patients with high and low pre-resection serum lactate concentrations.

Variable	High lactate (≥ 2 mmol/L)	Normal lactate (< 2 mmol/L)	p-value
Tumor grade, %			
High	56.8%	43.2%	
Low	21.1%	78.9%	< 0.01
Gender, %			
Male	33.0%	67.0%	
Female	57.8%	42.2%	< 0.01
Age (mean, SD)	50.8 (18.7)	47.7 (20.5)	0.34
Tumor size, %			
≥ 50 mm	47.4%	37.7%	
< 50 mm	52.6%	62.3%	0.27

SD – standard deviation.

surgery corticosteroid use and lactate levels, with a mean lactate level of 1.61 mmol/L in patients with corticosteroid use vs. 1.89 mmol/L in patients with no registered use of these drugs ($p = 0.04$).

In the univariate logistic regression, there was a significant association between elevated pre-resection serum lactate and high tumor grade (OR = 4.94; $p < 0.01$; AUC 0.70). A high tumor grade was also associated with older age (OR = 1.10; $p < 0.01$) and increased pre-surgery corticosteroid use (OR = 2.61; $p = 0.02$). There was no association between tumor size and sex with tumor grade (Table 3).

In the multivariate regression model, both older age, corticosteroid use, and elevated lactate retained their association with high tumor grade (Table 3), implying that, after adjusting for all other factors, increased lactate concentration are a predictor of high-grade gliomas. The OR for elevated pre-resection serum lactate increased from 4.94 to 14.33 after adjusting for age and corticosteroid, and the AUC for the final regression model was 0.98.

Table 3
Logistic regression analysis of the association between tumor grade and pre-resection serum lactate concentrations.

	Univariate logistic regression		Logistic regression model	
	Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
Lactate level (≥ 2 mmol/L)	4.94 (2.32–10.51)	< 0.01	14.33 (4.01–56.07)	< 0.01
Age, years	1.10 (1.06–1.13)	< 0.01	1.13 (1.08–1.17)	< 0.01
Corticosteroid use	2.61 (1.31–5.17)	0.02	9.49 (2.86–31.45)	< 0.01
Sex, male	1.59 (0.83–3.07)	0.16		
Tumor size ≥ 50 mm	1.19 (0.58–2.44)	0.63		

4. Discussion

Increased glucose uptake is a common feature of malignant tumor cells, with consequent production of lactic acid, even in aerobic conditions, and it is known that a lactate accumulation occurs in the GBM microenvironment [7,14]. Kohli-Seth et al provided the first evidence of the systemic repercussion of this phenomenon in a retrospective study reporting hyperlactacidemia in a large number of patients from an intensive care unit (ICU) submitted to brain tumor resection surgery, but they did not correlate these findings with tumor pathology [15]. In 2016, Kahlon et al. published a case of chronically elevated lactate levels in a patient with GBM as a circumstance not previously reported, revealing an unawareness of the scientific community to the serum lactate behavior in this type of cancer [16].

Two previous studies have aimed to find an association between serum lactate and gliomas malignancy [9,10]. Mariappan et al observed the pre-surgical lactate levels of 50 patients undergoing brain surgery for glioma resection and found an association between high lactate levels and high-grade tumors [9]. Shih C. et al, aimed to identify potential pretreatment serum biomarkers that might influence oncological outcomes in patients with primary brain tumor and found the same association [10].

In the present study, we found a significant association between high grade glioma and high levels of pre-surgical serum lactate. There is not a clear definition of the values from which serum lactate concentration is considered to be elevated, but most studies use cut-offs between 2.0 and 2.5 mmol/L [8]. Therefore, we defined values equal or above 2.0 mmol/L as "elevated". Our findings confirmed the results described in previous studies, but in a larger sample and assessing the effect of potential confounding factors not previously evaluated, which reinforces the strength of this hypothesis. However, we did not evaluate serum lactate levels at other phases or after surgery. Furthermore, we did not assess the role of serum lactate levels as a predictor of survival. A previous study by Cata et al. found no association between lactate levels and survival in gliomas, suggesting that serum lactate has probably more potential as a biomarker for diagnostic and staging than prognostic [17].

We excluded patients with known liver or renal dysfunction and with congestive heart failure, because these conditions are known to cause a rise in serum lactate. High-dose corticosteroid therapy could also potentially rise lactate levels [18] and a large number of patients with high grade glioma are under this type of therapy. Previous studies did not assess this factor. We reviewed the clinical files of our patients in order to determine if they underwent corticosteroid therapy before surgery and found that the effect of high lactate concentration in predicting high-grade gliomas is not changed after adjusting for the use of corticosteroid in the multivariable model. This finding suggests that the observed increase in mean lactate levels in patients with high grade gliomas is related to the tumor biology, and not to the use of corticosteroids. Nevertheless, we have to be careful in establishing a definitive conclusion. Given the retrospective nature of this review, we cannot exclude that information on corticosteroid therapy could be absent in some of the clinical files, particularly pre-admission use. Future prospective studies are warranted to indisputably resolve this issue, namely by systematically and per study protocol measuring lactate levels before any corticosteroid use in a consecutive group of patients.

These results are encouraging in what concerns to the usefulness of serum lactate determination in the diagnosis of gliomas, and they raise some additional questions and hypothesis that should be addressed in future studies. It would be interesting to compare serum lactate levels with MRI spectroscopy results, since it has been showed that malignant transformation of the glial tumors is accompanied by the presence of elevated lactate peaks in MR spectroscopy of high grade gliomas [19]. Indeed, an association between elevated lactate peak in MR spectroscopy and hyperlactacidemia in high-grade gliomas would support the

role of the tumor as the cause of the metabolic phenomenon leading to hyperlactatemia and, consequently, the potential of serum lactate as a biomarker of glioma malignancy. A panel of biomarkers for tumor malignancy, including lactate, could be probably more helpful than any single isolated measurement. Lactate-related enzymes, such as lactate dehydrogenase (LDH) are particularly promising. It is known that LDH-A, which catalyses the interconversion of pyruvate and lactate, is up-regulated in human cancers, including GBM [20]. Lutterbach et al. showed that raised serum LDH levels may negatively influence outcome in glioblastoma patients [21]. This findings suggest that LDH could be an interesting biomarker of glioma grade. Although LDH may be elevated in several situations, like hemolysis, thromboembolism, systemic infection and inflammation and muscle injury [22], it could potentially represent an additional and perhaps more sensitive tool than serum lactate in the diagnosis of high grade glioma. Additional studies are also warranted to determine if this method could also be suitable for the differential diagnosis between gliomas and other brain tumors, like metastasis and lymphomas

5. Conclusion

In all, this study reinforces the role of serum lactate as a potential biomarker of brain tumors malignancy, and its results encourage further research on this subject.

Ethical statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

References

- [1] A. Omuro, L.M. DeAngelis, Glioblastoma and other malignant gliomas: a clinical review, *JAMA* 310 (17) (2013) 1842–1850.
- [2] C. Adamson, O.O. Kanu, A.I. Mehta, et al., Glioblastoma multiforme: a review of where we have been and where we are going, *Expert Opin. Invest. Drugs* 18 (8) (2009) 1061–1083.
- [3] D.N.O.H. Louis, O.D. Wiestler, W.K. Cavenee, WHO Classification of Tumours of the Central Nervous System -Revision, 4th edition, WHO, 2016.
- [4] L. Zhou, H. Tang, F. Wang, et al., Bioinformatics analyses of significant genes, related pathways and candidate prognostic biomarkers in glioblastoma, *Mol. Med. Rep.* 18 (5) (2018) 4185–4196.
- [5] E.T. Sayegh, G. Kaur, O. Bloch, A.T. Parsa, Systematic review of protein biomarkers of invasive behavior in glioblastoma, *Mol. Neurobiol.* 49 (3) (2014) 1212–1244.
- [6] A. Mansouri, L.D. Hachem, S. Mansouri, et al., MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges, *Neuro Oncol.* (2018).
- [7] C.B. Colen, Y. Shen, F. Ghoddoussi, et al., Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: an in vivo study, *Neoplasia* 13 (7) (2011) 620–632.
- [8] L.W. Andersen, J. Mackenhauer, J.C. Roberts, K.M. Berg, M.N. Cocchi, M.W. Donnino, Etiology and therapeutic approach to elevated lactate levels, *Mayo Clin. Proc.* 88 (10) (2013) 1127–1140.
- [9] R. Mariappan, L. Venkatraghavan, A. Vertanian, et al., Serum lactate as a potential biomarker of malignancy in primary adult brain tumours, *J. Clin. Neurosci.* 22 (1) (2015) 144–148.
- [10] C.C. Shih, T.S. Lee, F.Y. Tsuang, et al., Pretreatment serum lactate level as a prognostic biomarker in patients undergoing supratentorial primary brain tumor resection, *Oncotarget* 8 (38) (2017) 63715–63723.
- [11] S. Bharadwaj, L. Venkatraghavan, R. Mariappan, et al., Serum lactate as a potential biomarker of non-glial brain tumors, *J. Clin. Neurosci.* 22 (10) (2015) 1625–1627.
- [12] R. Mariappan, L. Venkatraghavan, A. Vertanian, et al., Serum lactate as a potential biomarker of malignancy in primary adult brain tumours, *J. Clin. Neurosci.* 22 (1) (2015) 144–148.
- [13] A.L. Shetty, K. Thompson, K. Byth, et al., Serum lactate cut-offs as a risk stratification tool for in-hospital adverse outcomes in emergency department patients screened for suspected sepsis, *BMJ Open* 8 (1) (2018) e015492.
- [14] J. Li, S. Zhu, J. Tong, et al., Suppression of lactate dehydrogenase A compromises

- tumor progression by downregulation of the Warburg effect in glioblastoma, *Neuroreport* 27 (2) (2016) 110–115.
- [15] R. Kohli-Seth, S.R. Mukkera, A.B. Leibowitz, et al., Frequency and outcomes of hyperlactatemia after neurosurgery: a retrospective analysis, *ICU Dir.* 2 (6) (2011) 211–214.
- [16] A.S. Kahlon, M. Alexander, A. Kahlon, J. Wright, Lactate levels with glioblastoma multiforme, *Proceedings (Baylor University Medical Center)* 29 (3) (2016) 313–314.
- [17] J.P. Cata, S. Bhavsar, K.B. Hagan, et al., Intraoperative serum lactate is not a predictor of survival after glioblastoma surgery, *J. Clin. Neurosci.* 43 (2017) 224–228.
- [18] T.H. Ottens, M.W.N. Nijsten, J. Hofland, et al., Effect of high-dose dexamethasone on perioperative lactate levels and glucose control: a randomized controlled trial, *Crit. Care (London, England)* 19 (1) (2015) 41–41.
- [19] M. Bulik, R. Jancalek, J. Vanicek, A. Skoch, M. Mechl, Potential of MR spectroscopy for assessment of glioma grading, *Clin. Neurol. Neurosurg.* 115 (2) (2013) 146–153.
- [20] S. Daniele, C. Giacomelli, E. Zappelli, et al., Lactate dehydrogenase-A inhibition induces human glioblastoma multiforme stem cell differentiation and death, *Sci. Rep.* 5 (2015) 15556.
- [21] J. Lutterbach, W. Sauerbrei, R. Gittenberger, Multivariate analysis of prognostic factors in patients with glioblastoma, *Strahlenther. Onkol.* 179 (1) (2003) 8–15.
- [22] C. Perry, H. Peretz, O. Ben-Tal, A. Eldor, Highly elevated lactate dehydrogenase level in a healthy individual: a case of macro-LDH, *Am. J. Hematol.* 55 (1) (1997) 39–40.

5 ASSOCIATION BETWEEN RED BLOOD CELL DISTRIBUTION WIDTH AND SURVIVAL IN GLIOBLASTOMA PATIENTS

Paulo Linhares,^{1,2} Ana V. Ferreira, ^{1,2} Bruno Carvalho, ^{1,2} Rui Vaz ^{1,2}

¹ Neurosurgery Department, São João Hospital Centre, Oporto, Portugal; ² Faculty of Medicine of Oporto University, Oporto, Portugal.

ABSTRACT

Purpose: Several authors studied hematological markers as prognostic determinants in patients with neoplasia. The red blood cell distribution width (RDW) has been recently described as a possible indicator, although its study is still scarce in glioblastoma patients. Our purpose was to assess RDW as a prognostic factor of disease progression and overall survival in glioblastoma patients.

Methods: We analyzed a cohort of glioblastoma patients submitted to surgical resection for eight years. Data records regarding pre and post-surgery RDW were collected. Association with Progression-Free Survival (PFS) and Overall Survival (OS) was determined using Kaplan Meyer curves and Cox regression.

Results: Were assessed 140 patients, 98 men and 42 women, with an average age of 62.9 ± 10.00 years old, PFS median (IQR) of 7 (10), and OS of 16 (14) months. The average presurgery RDW was $13.0 \pm 0.72\%$, lower than post-surgery RDW, $14.1 \pm 1.33\%$, $p=0.021$. The exploratory analysis determined a presurgery cutoff of $\text{RDW} > 14.5\%$ with a more inferior PFS (7, 95%CI 5.26-8.73) vs. $\text{RDW} > 14.5\%$ (2, 95%CI 0.00-5.27), as well as OS – 7 (95%CI 0.0-15.8) vs. 16 (95%CI 14.8-17.2) months, respectively. Regarding univariate analysis, presurgery $\text{RDW} > 14.5\%$ showed an association with OS [HR 6.070 (95%CI 2.122-17.361), $p=0.001$] and a PFS [HR 3.242 (95%CI 1.165-9.026), $p=0.024$]. This association remained for OS when adjusted to related variables [HR 5.679 (95%CI 1.910-16.886), $p=0.002$]. Authors found no clinically relevant associations for post-surgical RDW regarding PFS nor OS.

Conclusion: Results support presurgery RDW as an independent prognostic factor for PFS and OS in glioblastoma patients.

Keywords: RDW, Glioblastoma, Progression-Free Survival, Overall Survival.

Declarations

Funding: We had no funding for the development of this research.

Conflicts of interest/Competing interests: There is no conflict of interest from any of the authors at the time of this submission or research.

Availability of data and material: Raw data collected is available for peer analysis.

Code availability: Not applicable.

Authors' contributions: Paulo Linhares and Ana Ferreira were responsible for the collection, analysis and interpretation of the data as well as the writing of the present manuscript. All authors contributed to the analysis and understanding of the data as well as for the revision and writing of the current document. All authors approved the final version of the manuscript.

Ethics approval: Authors obtained Local Ethics Committee approval before data collection.

Consent to Participate: Not applicable.

Consent to publication: All authors approved the final version of the manuscript for publication.

1. INTRODUCTION

Despite aggressive surgery and radiochemotherapy, prognosis in patients with glioblastoma (GBM) remains poor, with median overall survival estimated at 15 months. Among the different factors that can contribute to this result are, on the one hand, the diversity of processes involved in gliomagenesis and, on the other hand, the fact the disease is already in an advanced stage when symptoms appear. Several attempts have been made to find a blood marker or a set of markers that can be used in these patients' diagnosis and follow-up. So far, the potential biomarkers studied require complex determination processes and have little specificity. They translate processes associated with inflammatory, immunological, coagulation, vasculogenesis, or tumor growth changes. Prognostic factors may be of fundamental importance in determining the best therapeutic option, particularly in some subgroups of patients, such as the elderly and patients with multiple comorbidities. Red blood cell distribution width (RDW) estimates erythrocyte size variation calculated as the coefficient of variation. A higher RDW represents a broader red blood cells (RBC) size range, while a smaller value indicates size homogeneity^[1]. RDW is a systemic cellular inflammatory marker. Considering that the RDW analysis is part of the basic preoperative blood study, it becomes an easily accessible and universal variable. There is growing evidence that RDW can be a predictor of adverse events regardless of diagnosis. Thus, RDW is a promising prognostic inflammation marker for the clinical decision process, focusing on cancer outcomes^[2]. Former studies have conflicting results regarding the potential role of RDW as an independent risk factor for survival in GBM^[3,4]. This study aimed to assess RDW as a prognostic factor of survival in GBM patients.

2. METHODS

2.1. Study design and setting

Single-center, retrospective observational study for the analysis of RDW as a prognostic factor in patients with a histologically confirmed diagnosis of GBM submitted to surgery. The identified patients were part of a prospectively collected cohort database of patients diagnosed between 2005 to 2013 with a six-year follow-up through the year 2019. In our center, after surgery, patients are treated with standard chemoradiation therapy protocol. The work described has been carried out following the Declaration of Helsinki. The local ethics committee approved data collection and analysis. STROBE statement guidelines were used for the structure.

2.2. Clinical data collection

We retrospectively reviewed a prospectively maintained database of patients with cerebral tumors. We selected the patients with GBM submitted to resection surgery between January 2005 and January 2013 and reviewed all patients' clinical registries with a minimum of six-years follow-up. All demographic and clinical data from the database and clinical registries were collected. All patients met the following eligibility criteria: age >18 years; GBM diagnosed by histopathology; no previous chemotherapy or radiotherapy before surgery; no history of any other malignant disease. Patients who underwent tumor biopsy or had regrowth tumors as an initial diagnosis in this period were excluded.

2.3. Variables

RDW coefficient of variation, also known as RDW-CV, will be referred to as RDW. We registered RDW in two moments: immediately before surgery and in the first appointment before the beginning of adjuvant chemoradiotherapy or radiotherapy, representing 4 to 6 weeks after surgery.

Overall survival (OS), defined as the time from surgery to the time of death, was measured in months such as progression-free survival (PFS), defined as the time from surgery to tumor progression registered by magnetic resonance imaging. The diagnosis of GBM was defined by the histologic result from surgical resection according to the WHO Classification of SNC tumors.

Were considered as other variables for analysis: gender, age at diagnosis, tumor central location (frontal, temporal, parietal, occipital), tumor extension (one lobe, two lobes, more than two), tumor focality, eloquent areas involvement, comorbidities

able to influence RDW (hypertension, diabetes mellitus, metabolic syndrome, cardiac, renal or hepatic disease, inflammatory or auto-immune diseases), type of first-line adjuvant systemic therapy (STUPP or other), second-line adjuvant systemic therapy and surgery extent of resection.

2.4. Statistical Methods

Continuous variables are described as mean $\pm$ standard deviation or median and interquartile range (IQR) considering variable distribution. Periods for PFS and OS are represented as median and interquartile range or 95% confidence interval. T-student test for related samples was used for continuous intra-patients variable comparison.

Association analysis between RDW and survival, OS and PFS, were established by Kaplan-Meier Curves and Cox Regression for univariate and multivariate analysis. RDW cutoff was explored as the lowest level with a significant difference value in the Kaplan Meier curves for PFS and OS. All statistic tests were reported considering as statistically significant a p-value of 0.05. Statistical analysis was performed with IBM SPSS Statistics 25.0.

3. RESULTS

3.1. Patients

Were included 140 GBM submitted to resection surgery. All patients included had over 80% of the tumor volume removed. Demographic and clinical characteristics of the analyzed cohort are detailed in Table 1. The male-to-female ratio was 2.3:1. The mean age at surgery was 62.9 ± 10.0 years, and the mean age at death was 64.4 ± 9.8 years. The median overall survival rate was 19.4 ± 14.34 months, and the mean progression-free survival was 9.4 ± 8.73 months.

3.2. Main Results

The average presurgery RDW was $13.0 \pm 0.72\%$, with values ranging from 11.5-15.4%. Post-surgery RDW mean value was $14.1 \pm 1.33\%$ varying between 12.3 to 20.1%. When compared both values, there was a statistically significant difference with a p-value of 0.021.

Using a sequential analysis of RDW cutoffs by Kaplan-Meier curves, it was possible to identify that a presurgery RDW $>14.5\%$ had lower OS (7, 95%CI 0.0-15.8 months) when compared to those with RDW $\leq 14.5\%$ (16, 95%CI 14.8-17.2 months), $p < 0.005$, as may be seen in Figure 1 and Figure 2.

As for post-surgery RDW, patients with cutoff $>15\%$ had lower PFS (5, 95%CI 3.62-6.37) when compared to RDW $\leq 15\%$ (8, 95%CI 5.89-10.11), $p = 0.035$. However, there was no relation between post-surgery RDW and OS.

Based on previous findings, patients were divided into two groups, according to presurgery RDW cutoff of 14.5%. This cutoff was considered for Cox Regression univariate and multivariable models when analyzing the association between RDW, PFS (Table 2), and OS (Table 3).

The univariate analysis showed an association between presurgery RDW to OS [HR=6.126 (95%CI 2.142-17.521), $p = 0.001$] and PFS [HR=3.178 (95%CI 1.143-8.839), $p = 0.024$]. The relation to OS remained in the multivariate analysis when adjusted to statically significant related variables [HR=7.859 (95%CI 2.540-24.317), $p < 0.001$]. Although age did not have an established association, it was included in the model since it is referred in the literature as a factor that may influence RDW.

4. DISCUSSION

Inflammation is a hallmark of cancer^[5]. Inflammatory conditions promote cancer initiation and progression in various tissues being a critical process in gliomagenesis. RDW evolved as a prognostic marker of different medical conditions such as sepsis, acute myocardial infarction, heart failure, auto-immune, and liver diseases. Previous studies also indicated that increased RDW is associated with enhanced all-cause mortality in the general population^[6].

It is established that a strong relation between RDW and subclinical inflammatory states may relate to the tumor microenvironment^[7].

A meta-analysis of esophageal cancer, lung cancer, hepatocellular carcinoma, breast cancer, urothelial carcinoma, and specific types of hematologic cancer such as chronic lymphocytic leukemia indicates that elevated RDW significantly predicts poor survival outcomes for these patients^[2].

The role of peripheral blood inflammatory indicators in gliomas remains to be established^[8]. RDW elevation is correlated with an increase in inflammatory markers, such as soluble tumor necrosis factor receptors, interleukin-6, and C-reactive protein^[9].

A 2017 systematic review and meta-analysis by Hu et al. covered 16 articles and 4267 cancer patients^[2]. None of these articles referred to patients with glioblastoma. The combined results of this meta-analysis showed elevated RDW was associated with poor OS [HR=1.47, (95% CI: 1.29-1.66)] and PFS [HR=3.21, (95% CI: -0.33- 6.75)].

In the study of Liang R et al., RDW levels lower than 14.10% were associated with favorable OS, and Kaplan-Meier survival curves showed that patients belonging to the high-RDW group had a worse median OS (293 days versus 375 days, $p=0.023$) than those belonging to the low-RDW group^[4].

Wang DP et al. meta-analyses showed that patients with a higher RDW had lower OS rates [HR=1.40 (95% CI 1.13–1.74), $p=0.002$] and that increased RDW was found to be an independent predictor of worse survival in patients with gliomas. These results were in concordance with our study that showed that presurgery RDW is associated with OS [HR=6.070 (95%CI 2.122-17.361), $p=0.001$].

However, in a retrospective study of 219 glioma patients, Bao Y et al. reported no significant difference in the median OS between the high and low RDW groups for a cutoff of 12.5. The median survival in the high RDW group was 15 months (95% CI, 11.590 - 18.410) and was 23 months in the low RDW group (95% CI, 1.125 - 44.875; $P = 0.184$) [10]. This study's cutoff was inferior to most of the studies, which shows a cutoff over 14%, which could probably explain the lack of statistical significance. Our study showed that a 14.5% cutoff of preoperative RDW was associated with a worse prognosis. If we consider the postoperative values, we find a lower PFS with RDW levels over 15%.

One possible explanation for elevated RDW in cancer patients is the hypoxia state that stimulates erythropoietin. This is involved in expediting RBC production and stimulating reticulocytes' early release, leading to increased RDW^[11].

Another explanation is the associated inflammation impairment in erythrocyte maturation, oxidative stress, and cancer-driven cytokine release, leading to an increased anisocytosis^[6].

Also, high levels of RDW mirror underlying inflammatory and malnutrition conditions that weaken erythrocyte maturation and causes inadequate production of the hormone erythropoietin, malnutrition, or oxidative damage^[11].

Several prognostic factors have been identified, including methylation of the O6-methylguanine-DNA-methyltransferase gene (MGMT) promoter, mutation of isocitrate-dehydrogenase gene 1 (IDH1), younger age, and good clinical performance status^[12].

A gradual increase of RDW with age has been documented, and RDW tends to increase in parallel with age [13]. Since age is also an important prognostic factor in glioblastoma patients, it was considered for RDW analysis. Our study showed that the increase in RDW was related to lower overall survival regardless of age. In the Malmo Diet and Cancer Study with 26820 participants, in patients aged 45 years and older, Bornè et al. show that the subjects with higher RDW were significantly older than those with the lowest values^[14].

The preoperative inflammatory response is correlated with glioma grading^[8]. Although pretreatment RDW level can reflect both inflammatory and nutritional status, it would be more convincing if combined with other potential predictors, such as neutrophil to lymphocyte ratio (NLR) and prognostic nutritional index (PNI)^[15]. Since glioblastoma is an injury confined to the central nervous system with little systemic repercussion, malnutrition is not a problem in these patients at the time of diagnosis.

One of the things that remain to be explained is the relationship between the preoperative inflammatory state and overall survival, with no sequential assessment during the disease and the little interference that treatments have on overall survival. Another aspect that should be considered is the relationship between inflammatory markers and imaging findings, namely the extension of peritumoral edema. If the RDW was only related to a tumor's presence, the values one month after surgery in complete removal cases should be lower than the preoperative values. However, our study shows higher values, which might be explained by the inflammatory process induced by surgery, but the relationship between high levels and worse PFS remains unclear. Its serial study as a hematologic indicator of disease progression should be pursued.

Another important aspect is that these markers are nonspecific, appearing in various pathologies as oncological, inflammatory, infectious, thrombosis, diabetes, and cardiovascular diseases.

Regarding the extent of resection, it was considered a complete removal in the absence of contrast enhancement in the early postoperative magnetic resonance imaging (before 72 hours postoperative) and the remaining subtotal removals, taking into account that all patients had tumor resection greater than 85% of the tumor volume. Therefore, the extent of removal was not a factor associated with overall survival and disease progression.

The relationship between corticotherapy and the RDW value has not been studied. It is expected that patients who need a higher dose of corticosteroids would be those who had more edema, therefore a more significant preoperative inflammatory response. The use of corticosteroids preoperatively, by decreasing the inflammatory response, should consequently reduce the value of RDW, but this was not verified. On the other hand, greater preoperative edema was not related to progression-free survival in several studies.

It has not been established whether an increased value of RDW is a risk factor, a consequence of the inflammation underlying the tumor, or only an epiphenomenon of an underlying biological and metabolic imbalance.

Limitations

One limitation is the retrospective design of the analysis, but this is partially overcome by the accuracy of the assessed items in the database, given its prospective collection characteristics.

Another significant limitation is the lack of research on the MGMT promoter gene's methylation, which is an important prognostic factor. However, we are not aware

of any relationship between the methylation of the MGMT promoter gene and the value of RDW. Preoperative and early postoperative corticosteroid therapy was also not quantified.

Conclusions

The results support the presurgery RDW over 14.5% as possible independent prognostic factor for OS in patients with glioblastoma. Despite the results obtained, the RDW results' interpretation must be made carefully because of the many clinical applications. Its conjunction with other hematological and imaging parameters may point out the prognosis of the disease in a more consistent way. The serial evaluation of a panel of biomarkers, including RDW throughout the disease, which are easy to access and interpret, may help in the earlier diagnosis of recurrence in a still pre-clinical and pre-imaging stage.

REFERENCES

1. Horne BD, Muhlestein JB, Bennett ST, Muhlestein JB, Ronnow BS, May HT, Bair TL, Anderson JL (2015) Association of the dispersion in red blood cell volume with mortality. *Eur J Clin Invest* 45 (6):541-549. doi:10.1111/eci.12432
2. Hu L, Li M, Ding Y, Pu L, Liu J, Xie J, Cabanero M, Li J, Xiang R, Xiong S (2017) Prognostic value of RDW in cancers: a systematic review and meta-analysis. *Oncotarget* 8 (9):16027-16035. doi:10.18632/oncotarget.13784
3. Auezova R, Ryskeldiev N, Doskaliyev A, Kuanyshev Y, Zhetpisbaev B, Aldiyarova N, Ivanova N, Akshulakov S, Auezova L (2016) Association of preoperative levels of selected blood inflammatory markers with prognosis in gliomas. *Onco Targets Ther* 9:6111-6117. doi:10.2147/OTT.S113606
4. Liang R, Li M, Yang Y, Mao Q, Liu Y (2017) Significance of Pretreatment Red Blood Cell Distribution Width in Patients with Newly Diagnosed Glioblastoma. *Med Sci Monit* 23:3217-3223
5. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144 (5):646-674. doi:10.1016/j.cell.2011.02.013
6. Goyal H, Hu ZD (2017) Prognostic value of red blood cell distribution width in hepatocellular carcinoma. *Ann Transl Med* 5 (13):271. doi:10.21037/atm.2017.06.30
7. Salvagno GL, Sanchis-Gomar F, Picanza A, Lippi G (2015) Red blood cell distribution width: A simple parameter with multiple clinical applications. *Crit Rev Clin Lab Sci* 52 (2):86-105. doi:10.3109/10408363.2014.992064
8. Wang DP, Kang K, Lin Q, Hai J (2020) Prognostic Significance of Preoperative Systemic Cellular Inflammatory Markers in Gliomas: A Systematic Review and Meta-Analysis. *Clin Transl Sci* 13 (1):179-188. doi:10.1111/cts.12700
9. Forhecz Z, Gombos T, Borgulya G, Pozsonyi Z, Prohaszka Z, Janoskuti L (2009) Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. *Am Heart J* 158 (4):659-666. doi:10.1016/j.ahj.2009.07.024
10. Bao Y, Yang M, Jin C, Hou S, Shi B, Shi J, Lin N (2018) Preoperative Hematologic Inflammatory Markers as Prognostic Factors in Patients with Glioma. *World Neurosurg* 119:e710-e716. doi:10.1016/j.wneu.2018.07.252
11. Luo L, Quan X, Song X, Zhang L, Yin Y, He Q, Cai S, Zeng J, Zhang Q, Gao Y, Yu S (2017) Red blood cell distribution width as a predictor of survival in nasal-type, extranodal natural killer-T-cell lymphoma. *Oncotarget* 8 (54):92522-92535
12. Pierscianek D, Ahmadipour Y, Michel A, Chihi M, Oppong MD, Kebir S, Glas M, Stuschke M, Sure U, Jabbarli R (2020) Preoperative Survival Prediction in Patients With Glioblastoma by Routine Inflammatory Laboratory Parameters. *Anticancer Res* 40 (2):1161-1166. doi:10.21873/anticancer.14058
13. Patel KV, Ferrucci L, Ershler WB, Longo DL, Guralnik JM (2009) Red blood cell distribution width and the risk of death in middle-aged and older adults. *Arch Intern Med* 169 (5):515-523. doi:10.1001/archinternmed.2009.11
14. Borne Y, Smith JG, Melander O, Engstrom G (2014) Red cell distribution width in relation to incidence of coronary events and case fatality rates: a population-based cohort study. *Heart* 100 (14):1119-1124. doi:10.1136/heartjnl-2013-305028
15. Wang PF, Song SY, Guo H, Wang TJ, Liu N, Yan CX (2019) Prognostic role of pretreatment red blood cell distribution width in patients with cancer: A meta-analysis of 49 studies. *J Cancer* 10 (18):4305-4317. doi:10.7150/jca.31598

TABLES AND FIGURES

Table 1. Demographic and clinical data.

Parameters		
n		140
Age (years)		65 (15)
Gender	Men	98 (79%)
	Women	42 (30%)
KPS		82 (10)
OS (months)		16 (14)
PFS (months)		7 (10)
Tumor Location	Temporal	41 (29.3%)
	Frontal	34 (24.3%)
	Other	65 (46.4%)
Tumor Extension	1 Lobe	88 (62.9%)
	2 Lobes	46 (32.9%)
	>2 lobes	6 (4.3%)
Focality	Unifocal	121 (86.4%)
	Multifocal	29 (13.6%)
Involvement of Eloquent Areas	Yes	63 (45%)
	No	76 (54.3%)
Tumor Resection	Subtotal	82 (58.6%)
	Total	58 (41.4%)
First-line Systemic Treatment	STUPP	117 (83.6%)
	Other	9 (6.4%)
	None	14 (10%)
Second-line Systemic Treatment	Yes	64 (45.7%)
	No	76 (54.3%)
RDW presurgery (%)		13.0±0.72 (11.5-15.4)
RDW post-surgery (%)		14.1±1.33 (12.3-20.1)

Continuous variables are presented as mean ± standard deviation (min-max) or mean (IQR) and categorical variables as absolute (relative) frequencies.

OS overall survival; **PFS** progression-free survival; **KPS** Karnofsky Performance Status.

Table 2. Cox Regression for Progression Free Survival.

Progression-free Survival	Univariate	p	Multivariate	p
Sex	0,952 (0,632-1,355)	0,690	1,007 (0,986-1,028)	0,530
Age	1,023 (1,006-1,041)	0,009*		
KPS	0,172 (0,023-1,270)	0,084		
Uni or Multifocal	2,093 (1,253-3,495)	0,005*	2,112 (1,203-3,708)	0,009*
Eloquent Areas Involvement	1,155 (0,815-1,636)	0,417		
Tumor Extension	2,898 (1,164-7,210)	0,022*	4,026 (1,584-10,235)	0,003*
Comorbidities	1,608 (1,119-2,311)	0,010*	1,575 (1,069-2,319)	0,022*
First Line treatment (STUPP)	2,823 (1,657-4,808)	<0,001*	2,890 (1,624-5,145)	<0,001*
Second Line treatment	0,696 (0,493-0,983)	0,040*	0,989 (0,633-1,546)	0,962
Surgery resection extent	0,849 (0,594-1,196)	0,339		
RDW Before Surgery (14.5%)	3,178 (1,143-8,839)	0,027*	2,581 (0,871-7,650)	0,087
Leukocytes	0,987 (0,934-1,043)	0,633		

*Significance value $p < 0.05$ **Table 2.** Cox Regression for Overall Survival.

Overall Survival	Univariate	p	Multivariate	p
Sex	1,001(0,691-1,449)	0,997		
Age	1,024 (1,006-1,041)	0,007*	1,011 (0,990-1,032)	0,304
KPS	0,43 (0,02-0,951)	0,046*	239202 (0,0-1,0,70e183)	0,953
Uni or Multifocal	1,973 (1,199-3,247)	0,007*	1,626 (0,861-1,955)	0,086
Eloquent Areas Involvement	1,603 (1,131-2,271)	0,008*	1,283 (0,850-1,939)	0,236
Tumor Extension	1,415 (0,577-3,471)	0,449		
Comorbidities	1,53 (1,078 – 2,176)	0,017*		
First Line treatment (STUPP)	2,618 (1,629-4,207)	<0,001*	3,626 (1,990-6,606)	<0,001*
Second Line treatment	0,710 (0,504-1,001)	0,050*	0,934 (0,594-1,468)	0,766
Surgery resection extent	0,766 (0,541-1,085))	0,134		
RDW Before Surgery (14.5%)	6,126 (2,142-17,521)	0,001*	7,859 (2,540-24,317)	<0,001*
Leukocytes	1,025 (0,975-1,078)	0,336		

*Significance value $p < 0.05$

Figure 1. Kaplan Meier Overall Survival Curve for RDW 14.5% before surgery.

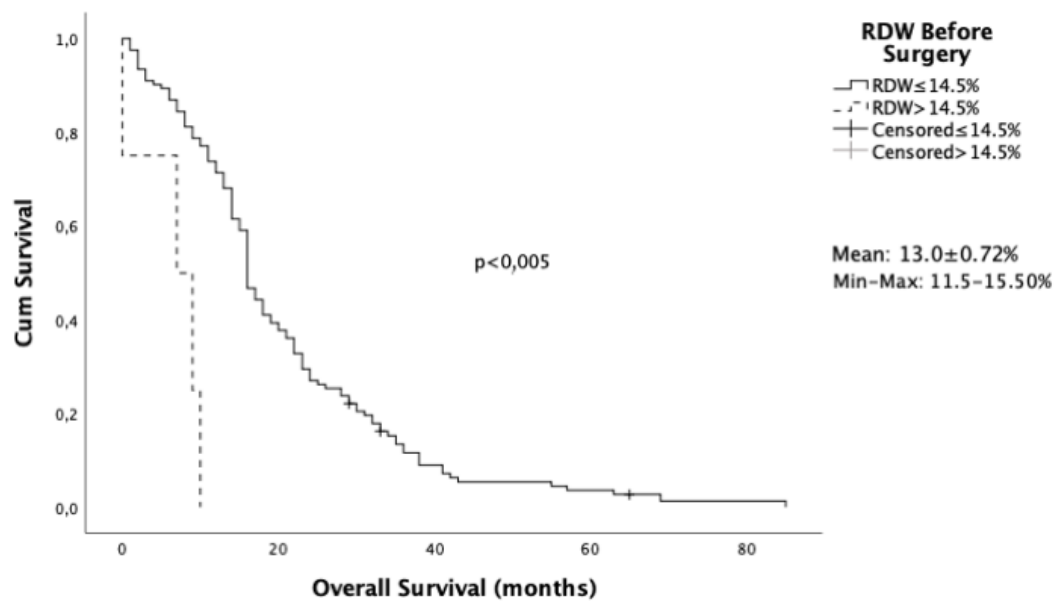
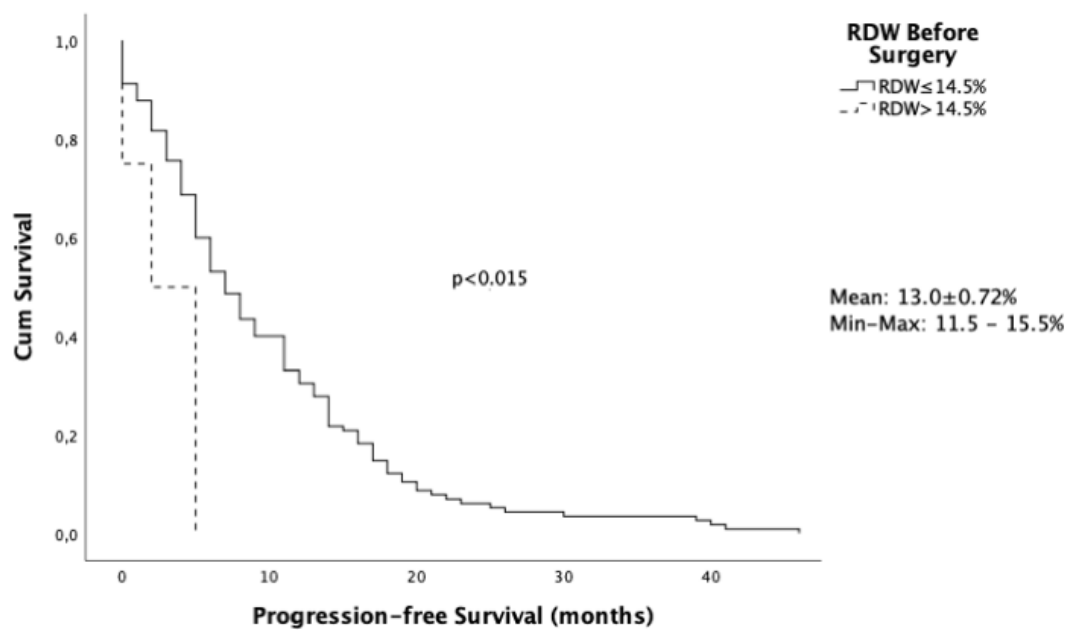


Figure 2. Kaplan Meier Progression-Free Survival Curve for RDW 14.5% before surgery.



6 GLIOBLASTOMA: IS THERE ANY BLOOD BIOMARKER WITH TRUE CLINICAL RELEVANCE?

Paulo Linhares,^{1,2} Bruno Carvalho,^{1,2} Rui Vaz,^{1,2} and Bruno M Costa^{3,4}

¹Neurosurgery Department, Centro Hospitalar São João, Porto, Portugal; ²Clinical Neurosciences and Mental Health Department, Faculty of Medicine, University of Oporto, Porto, Portugal; ³Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho; ⁴ICVS/3B's - PT Government Associate Laboratory, Braga, Portugal



Review

Glioblastoma: Is There Any Blood Biomarker with True Clinical Relevance?

Paulo Linhares ^{1,2,*}, Bruno Carvalho ^{1,2,*}, Rui Vaz ^{1,2} and Bruno M. Costa ^{3,4}

¹ Neurosurgery Department, Centro Hospitalar São João, Alameda Prof Hernani Monteiro, 4200-319 Porto, Portugal; paulojlinhares@yahoo.com (P.L.); ruimcvaz@gmail.com (R.V.)

² Clinical Neurosciences and Mental Health Department, Faculty of Medicine, University of Oporto, Alameda Prof. Hernani Monteiro, 4200-319 Porto, Portugal

³ Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; bfmcosta@med.uminho.pt

⁴ ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, 4710-057 Braga, Portugal

* Correspondence: bmfcavalho@gmail.com; Tel.: +35-19-6451-8544

Received: 21 July 2020; Accepted: 10 August 2020; Published: 13 August 2020



Abstract: Glioblastoma (GBM) is the most frequent malignant primary brain tumor in adults, characterized by a highly aggressive, inflammatory and angiogenic phenotype. It is a remarkably heterogeneous tumor at several levels, including histopathologically, radiographically and genetically. The 2016 update of the WHO Classification of Tumours of the Central Nervous System highlighted molecular parameters as paramount features for the diagnosis, namely *IDH1/2* mutations that distinguish primary and secondary GBM. An ideal biomarker is a molecule that can be detected/quantified through simple non- or minimally invasive methods with the potential to assess cancer risk; promote early diagnosis; increase grading accuracy; and monitor disease evolution and treatment response, as well as fundamentally being restricted to one aspect. Blood-based biomarkers are particularly attractive due to their easy access and have been widely used for various cancer types. A number of serum biomarkers with multiple utilities for glioma have been reported that could classify glioma grades more precisely and provide prognostic value among these patients. At present, screening for gliomas has no clinical relevance. This is because of the low incidence, the lack of sensitive biomarkers in plasma, and the observation that gliomas may develop apparently de novo within few weeks or months. To the best of our knowledge, there is no routine use of a serum biomarker for clinical follow-up. The purpose of this paper is to review the serum biomarkers described in the literature related to glioblastoma and their possible relationship with clinical features.

Keywords: glioblastoma; biomarkers; prognosis; diagnosis

1. Introduction

Glioblastomas (GBMs) are the most frequent primary malignant brain tumor, accounting for approximately 15% of all intracranial neoplasms and 45–50% of all primary malignant brain tumors [1]. The clinical behavior of individual tumors of a specific histopathological entity may differ substantially. Thus, additional markers are needed for a more refined and objective glioma classification, a better prediction of prognosis, and tailored therapeutic decision-making [2]. The revised 2016 World Health Organization (WHO) classification of tumors of the central nervous system recognizes the molecular evaluation as critical for the classification, since the mutation status of the *IDH1* and *IDH2* and the codeletion of 1p/19q are crucial for diagnosis, and the *TERT* promoter gene mutations and *ATRX* alterations are associated with prognosis. However, other molecular alterations may be associated with diagnosis, prognosis, and glioma risk.

A “biomarker” (biological marker) can be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention” [3]. The critical hallmark of a biomarker is to be highly sensitive and specific in providing information relevant for diagnosis, prognosis, or therapy [4]. Biomarkers should have the potential to identify cancer patients at an early stage, select patients who are most likely to benefit from therapy, and to guide the development of more effective agents [5]. The most useful tumor marker should possess high specificity and sensitivity to the tumor, and it should be unique.

GBMs show complex chromosomal and genetic alterations that lead to inactivation of various tumor suppressor genes, as well as aberrant activation of proto-oncogenes [2]. Glioblastoma *IDH*-wildtype lacks *IDH1/2* mutations and is considered primary glioblastoma. The typical genetic alterations of these tumors include *TERT* promoter mutations, homozygous deletion of *CDKN2A/CDKN2B*, loss of chromosomes 10p and 10q, *EGFR* alterations, *PTEN*, *TP53* and *PI3K* mutations [1]. Glioblastomas *IDH*-mutant show mutations in either the *IDH1* or *IDH2* gene and are characterized by frequent mutations in the *TP53* and *ATRX* genes, loss of 10q [1], and lack *EGFR* amplification [2]. They are mostly secondary, derived from lower-grade lesions. Often extensive tumor progression occurs in the interval between different imaging studies and/or goes undetected from lack of contrast enhancement. An alternative measure or marker of tumor burden may potentially permit earlier detection of treatment failure and allow for more timely therapeutic changes [6]. Single-molecule markers have been mostly insufficient to properly follow the dynamics of diseases like cancer and should be replaced by multiple marker profiles. The first study that systematically reviewed the diagnostic serum profile in GBM patients was published by Elstner et al. in 2011 and analyzed the serum concentrations of 14 proteins [7]. Nevertheless, no reliable marker has yet been identified for use in the diagnosis and surveillance of patients with malignant gliomas [8].

This review aims to summarize the potential of peripheral blood markers described in the literature and to critically evaluate its applicability to clinical practice.

2. Materials and Methods

A thorough search of the literature was performed using the MEDLINE database and Cochrane Reviews (January 2005 through December 2018) using the terms “glioma, serum biomarkers”. Articles in languages other than English were excluded. Only studies on data collected in humans were considered.

We divided the biomarkers into particular categories, although several of them have multiple roles and these categories are highly interrelated: (a) cell growth and vascular proliferation; (b) immune system; (c) inflammation; (d) nutritional status; and (e) coagulation markers.

3. Results and Discussion

The MEDLINE search identified 208 articles from January 2005 through December 2018, using the terms “glioma, serum biomarkers”. Articles in languages other than English were excluded, remaining 200 articles. Only studies on data collected in humans were considered. That gives a total 174 articles. Of these, only 39 articles specifically addressed potential serum biomarkers, 30 prospective studies, 5 retrospective studies and 4 revision studies, corresponding to 52 different molecules and 30 of them are referred only once and were included for final evaluation. Cochrane Reviews did not identify any title.

The most relevant potential serum biomarkers found in literature are summarized in Table 1. Many of these biomarkers belong to more than one category confirming the interaction between different hallmarks and cancer pathways. Although these biomarkers were closely related to each other, for a better text organization we describe the biomarkers by groups and discuss their potential roles in predicting the presence or the progression of glioblastoma.

Table 1. Serum biomarkers reported in the literature with potential application in glioblastoma.

Vascular Proliferation	Cell Growth	Inflammatory	Immune System	Coagulation	Nutritional
BMP-2	α 2-HS glycoprotein	CD23	CD14	Fibrinogen	IGFBP-2
CXCL 10	BMP-2	Albumin	CD23	ETG	Albumin
Haptoglobin α 2	Cathepsin D	Ratio N-L	IgE	Prothrombin factors	
Metalloproteinase 9	CXCL10	Ratio P-L	Osteopontin	TF (Tissue factor)	
Osteopontin	EGFR	Amyloid A1	Recoverin		
YKL-40	GFAP		TGF- β		
VEGF	Metalloproteinase 9		TNF- α		
PDGF-b	Osteopontin		IL-6		
TGF- β	Recoverin				
TNF- α	PDGF-b				
TSP1	IGF-1				
	TGF- β				
	TNF- α				
	IL-8				

3.1. Biomarkers Related to Vascular Proliferation and Cell Growth

Microvascular proliferation is a hallmark feature in glioblastoma. Being a complex process, multiple mechanisms are involved. Several biomarkers were studied in this context.

VEGF is the most important mediator of neovascularization in gliomas [9]. Studies measuring VEGF concentration in the serum of patients with glioblastoma have obtained conflicting results [10].

Salven et al. found serum levels of VEGF to be elevated in high-grade brain tumors [11]. In Reynés et al. work, in a series of 40 glioblastoma patients, serum levels of VEGF were twofold higher in patients compared with healthy controls [10]. Only pre-operative samples were collected, so VEGF could not be used as a follow-up biomarker. Stockhammer et al. found no differences between serum VEGF concentration in 6 patients with glioblastoma and in healthy controls [12]. Fine et al. did not find any correlation between serum VEGF levels and tumor response progression, time to progression, or overall survival [13]. Rafat et al. showed that levels of serum VEGF and granulocyte-macrophage colony stimulating factor (GM-CSF) are raised in patients with glioblastoma when compared with healthy controls [14]. Due to the lack of consistency in the different studies, some with very few cases, VEGF has not been shown to be a diagnostic or survival marker.

YKL-40 is a 40kDa glycoprotein produced by cancer, inflammatory and stem cells. It probably has a role in cell proliferation and differentiation, inflammation, protection against apoptosis, stimulation of angiogenesis, and regulation of extracellular tissue remodeling [5], and stimulates fibroblasts surrounding the tumor, although in vivo proof of these hypotheses is yet to be obtained [3]. Elevated serum YKL-40 is found in a subgroup of patients with different types of solid tumors [3]. Plasma levels of YKL-40 are highly correlated with age [5], and as plasma YKL-40 increases with age, levels in patients before or during treatment should be evaluated in comparison with age-stratified or age-adjusted reference levels [15]. YKL-40 is over-expressed in glioma tissues but is not expressed in normal brain or normal human astrocytes, suggesting that it is related to glioma initiation and progression [16] and may promote radiation and apoptosis resistance, increased invasiveness, and increased 72 KDa metalloproteinase activity [17]. It was first described as a potential serum biomarker in glioblastoma in 2002 by Tanwar [6], who found that the YKL-40 protein was dramatically elevated in a subset of the GBMs, and essentially below the detection limit for Western blot analysis, in both lower-grade tumors and normal brain extract. Statistically significant differences were detected between YKL-40 serum levels in patients with glioblastoma compared with controls and compared with patients with lower-grade gliomas, as well as between lower-grade tumors compared with controls but no significant differences were detected between high versus low tumor burden in GBM patients. In the series of Hormigo et al., tumor resection resulted in transiently increased serum levels of YKL-40 and reached a peak 24 h after resection [18]. These authors also found a significant difference in YKL-40

values for GBM patients with a complete resection compared with patients with evidence of disease. There was a significant association between the actual value of serum YKL-40 and survival. A low absolute YKL-40 value identified a subset of patients that is more likely to do well. Those patients with persistently normal values had a longer survival and disease-free interval [18]. In the same line of evidence, Bernardi et al. showed that YKL-40 serum levels determined at the 1st week after surgery may be a biomarker of outcome for high-grade gliomas. Despite a wide range of variability in YKL-40 levels, serum levels are higher in GBM patients than in healthy subjects, and surgery has a paramount influence on postoperative YKL-40 serum levels, raising the hypothesis that it may be a putative biochemical marker of extent of tumor resection [19]. Different data was showed by Linde et al. who found very little variation in YKL-40 protein concentrations in serum of patients throughout the disease course [20], and did not observe any correlation between serum YKL-40 and extent of resection or tumor volume. A possible explanation is the short follow-up and the time points of the serum collection. Besides that, no pre-operative values were collected and there were no correlations between these two data. There was no age adjustment nor correlation with tumor tissue expression. In spite of this data, Johansen conclude that there is still insufficient evidence to support its value outside of clinical trials as a screening tool, prognosticator of survival, predictor of treatment response and as a monitoring tool in the routine management of individual patients with cancer [21]. However, YKL-40 is one of the serum biomarkers with more evidence from different groups in GBM, and more robust further studies, with a larger number of patients with better characterization and new quantification techniques will be able to clarify and establish YKL-40 as a true clinically relevant GBM biomarker.

Epidermal growth factor receptor (*EGFR*) is a transmembrane cell surface receptor and a member of the c-erb-B family of tyrosine kinases, known to be overexpressed in a variety of human malignant tumors [22]. *EGFR* pathway plays prominent roles in regulating cell growth, apoptosis, and differentiation. Aberrant *EGFR* signaling may ultimately affect major hallmarks of cancer, including tumor growth, invasion, malignancy, and prognosis [23]. Induction of *EGFR* expression might be involved in the malignant transformation of human brain cells [24].

Choi et al. found that an overexpression of *EGFR* appears to correlate with glioma grade, which is observed in about 40–50% of GBMs compared to 10–26% of anaplastic astrocytomas [25]. *EGFR* gene is amplified in approximately 30–50% of malignant gliomas [26,27]. In the literature, results for the influence of *EGFR* expression on survival according to tissue immunohistochemical staining differ among researchers [25], despite the majority of papers showing a worse prognostic in patients with overexpression of *EGFR*. *EGFR* expression is also correlated with a poor response to radiation therapy [28]. While these results are related to tumor tissue expression, the associations between *EGFR* expression in tissue samples and in blood samples are yet to be demonstrated. Quaranta et al. showed the mean serum *EGFR* level in brain cancer patients is significantly elevated compared with that of healthy controls, with 73.8% of the patients demonstrating an elevated serum level of *EGFR* [26]. They concluded that serum *EGFR* extracellular domain may be potentially useful as a biological marker of gliomas for prediction of prognosis and follow-up after treatment.

GFAP is a 55kDa intermediate filament protein that is highly expressed in astrocytes as part of cytoskeleton [29] and may serve as an organ-specific marker [30]. The first description of serum GFAP in primary brain tumors patients was made by Brommeland et al. in 2007, including 27 GBM and four anaplastic astrocytoma patients. In a multivariate linear regression analysis, the only significant association was between pre-operative GFAP levels and tumor size. No difference was found between glioblastomas and astrocytomas [30], but the sample size was very small to allow a definitive conclusion. Although putting the possibility that GFAP values may reflect the evolution of the disease or the response to treatments, this extrapolation cannot be done because only the preoperative values were evaluated. Jung et al. studied a group of 50 patients with glioblastoma and found higher levels of serum GFAP compared to non-GBM tumor patients and healthy controls. In addition, serum GFAP concentration was closely linked with GBM tumor volume and tumor necrosis volume. As no follow-up assessment of serum GFAP was done, the only conclusion was that serum GFAP could be a diagnostic

biomarker of glioblastoma [31]. Still, the number of patients was too small ($n = 50$), and in 25% of GBM patients, no detectable levels of serum GFAP were found. Ilhan-Mutlu et al. found that GFAP plasma detectability was strongly associated with diagnosis of GBM in a series of 105 brain tumor patients, but it was only present in 38% of GBM patients (13/34). In these patients, no correlations were found between serum GFAP and tumor volume or survival [32]. It is another small series to allow reliable results. Husain H et al. evaluated the plasma GFAP levels before and after tumor debulking in a series of 33 gliomas. Detectable levels were found in 52% of patients pre-operatively and in 96% of patients post-operatively. In the GBM sub-group (nine patients), three having lower levels and two having higher levels was the main difference to the non-GBM group. Although they found a correlation with tumor grade and serum GFAP levels, the few number of patients enrolled again prevents reliable conclusions [29]. In a more recent prospective study of 33 GBM cases, 14 had elevated GFAP serum concentrations prior to any intervention [33]. Following surgery, all positive patients showed decreasing serum concentrations. An interesting factor was that 16 patients (48%) only did a stereotactic biopsy without reducing the tumor burden. There was no plausible explanation for this. Vietheer et al. concluded that serum GFAP at diagnosis are not predictive for survival or tumor recurrence.

An important bias is the small number of patients in each study and in the set of studies, precluding solid conclusions about the diagnostic, prognostic and follow-up relevance of GFAP in GBM.

Thrombospondin-1 (TSP-1) has tumor-inhibitory properties and inhibits tumor angiogenesis, however, no difference was found in serum samples between glioma patients and healthy controls [10]. Elstner et al. found a significant difference in serum protein concentration of TSP-1 between patients who survived more or less than 15 months [7]. Analysis of TSP1 alone predicts the survival chance for 80% of the GBM patients, but in conjunction with HSP70 and IGFBP3 the predictability increased to 100% [7]. Reynés et al. did not find any correlation with prognosis and survival. No further studies have been conducted with serum TSP-1 in the glioma context. Few studies and controversial results make it difficult to conclude the relevance of this putative biomarker.

Osteopontin (OPN) is a multifunctional arginine-glycineaspartate (RGD)-containing glycoposphoprotein with cytokine and chemokine properties [34] and has been implicated in tumor progression of a variety of systemic cancers [35]. Expression levels of osteopontin have been correlated with malignancy in human glioma cells and are associated with astrocytoma progression [36]. High cytoplasmic OPN expression in glioblastomas was associated with poor patients' survival [37]. Sreekanthreddy et al. carried out a detailed analysis of OPN at RNA and protein levels in tumor tissue and serum in different grades of astrocytoma as well as its effect on patient survival [38]. Comparisons of serum osteopontin levels were significant between GBM and normal controls, GBM and anaplastic astrocytomas, and GBM and diffuse astrocytomas. In a set of 30 newly diagnosed GBM patients they found that serum OPN levels > 20 ng/mL significantly correlate with the survival.

Recoverin (calcium-binding protein p26) is a cytoplasmic calcium-binding protein. The highest levels of expression are seen in the vitreous humor of the eye, but it can also be detected at low levels in the serum of normal adults [8]. Thirkill et al. first identified recoverin as a protein that binds antibodies expressed in patients with cancer-associated retinopathy (CAR), a paraneoplastic syndrome [39]. Sampath et al. found that serum recoverin levels were increased 10-fold in patients with active recurrent GBM compared with normal controls, and patients with quiescent GBM have only a slight increase [8]. The authors concluded that recoverin may be a useful glioma tumor marker, especially for recurrent active disease [8]. The sample only includes 5 patients with active recurrent disease and 2 with no signs of residual or recurrent disease so these conclusions are no more than extemporaneous, and no further studies could definitively confirm these findings. Manley et al. also investigated pediatric patients with glial tumors, and even though there was a correlation with progressive disease and elevated recoverin (A-PROTEIN) levels, the sensitivity and specificity of recoverin as a predictor of disease progression was insufficient to warrant its clinical use [40].

Cathepsin D may be involved in several actions that facilitate tumor progression [41]. Cathepsin D is expressed widely in the brain [42] and was confirmed to be upregulated in high-grade glioma [43]. The high expression of cathepsin D is one of the common features in the tissue samples of the high-grade astrocytomas [41]. Fukuda et al. analyzed the correlation between the molecular information and the clinical data of 78 glioma patients, including tumor grade, invasive nature and survival time. The serum levels of cathepsin D protein were compared between nine patients with low-grade gliomas and 11 patients with high-grade gliomas. The values were significantly higher in high-grade gliomas than in the low-grade tumors [41]. These data suggested that serum cathepsin D may be a potential indicator of the biological aggressiveness of intracerebral gliomas [41], but again, the very small number of patients is not reliable for definitive conclusions.

Cytokines are a group of multi-functional signaling proteins linked to angiogenesis, cell-growth, stem cell differentiation and regulation of immune responses, including tumor immune surveillance and tumor-induced immunosuppression [44]. In a group of 55 brain tumor patients, including 14 malignant, 17 benign and 24 metastatic Loh J-K show that pre-operative TGF- β 1 plasma levels were higher than in the control group. They also found significant decreases in TGF- β 1 level in benign and malignant tumors after removal. A slight but significant increase was seen in the metastatic group after surgery. These data allow the distinction between primary and metastatic tumors, but revealed no differences between the different primary tumors, in addition to only 6 being glioblastomas [45].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor- β (TGF- β) superfamily. The role of BMP2 in glioblastoma has not yet been elucidated [7], but appears to inhibit growth in gliomas [46], and is linked to angiogenesis and stem cell differentiation. BMP-2 expression in tumor tissue was significantly associated with prognosis [47]. Elstner studied the potential role of 14 serum secreted proteins, including BMP2 in a series of 23 GBM patients and 12 control subjects and concluded that the presence of a glioblastoma was only distinguished by serum concentrations of BMP2, CXCL10 and HSP70 in conjunction [7]. Nijaguna M, in a series of 148 GBM patients and 26 healthy controls, found a set of 18 cytokines to be different between the two groups [48]. Only pre-operative samples were collected, which prevents its use as a prognostic or follow-up marker. Schwartzbaum et al. analyzed serum samples from 487 subjects who were subsequently diagnosed with glioma [44]. This was the first study trying to find pre-diagnostic markers related to glioma risk or to the presence of a preclinical tumor. They have identified five serum cytokines and a cytokine-soluble receptor interaction, each associated with changes in the preclinical risk of glioma, but the authors themselves described some important bias and the results should be evaluated very carefully. These biases are transversal to this type of studies and are related to short follow-up times and small series of patients, with the inherent statistical limitations and implications in the results.

Peripheral IL-8 secretion correlates with its levels in astrocytic tumor tissues [49]. Ilhan-Mutlu et al. found an inverse association of IL-8 plasma concentration with tumor grade in glioma [32]. Elstner et al. show a reduced serum concentration for CXCL10 in glioblastoma patients but they did not find any correlation with the prognosis, tumor size or survival [7]. In a proteomic study with 35 GBM patients and 30 healthy controls, Popescu et al. found three candidate biomarkers for GBM diagnosis, CXCL4, S100A8 and S100A9 [50], but altered levels of these markers were also found in a set of different pathologies and were not specific to GBM.

MMP-9 is the most common metalloproteinase associated with neovascularization of tumors [51]. Expression of MMP-9 has been detected both in tumor and endothelial cells and is overexpressed in glioma. It could play an important role in promoting cell invasion into the surrounding brain and in glioblastoma resistance to antiangiogenic treatment [52]. Raithatha found that increasing levels of MMP-9 in glioma specimens correlates with higher tumor grade [53]. This correlation was also investigated in peripheral blood [18,54]. Hormigo et al. found a significant difference in MMP-9 values for GBM patients with a complete response compared with GBM patients with radiographic evidence of tumor in a series of 30 patients. However, the authors did not find correlations with tumor grade or overall survival [18]. Iwamoto et al. from the same group, and some years later, did not find any

correlation with serum MMP-9 levels and tumor size, survival or chemotherapy response, concluding that serum MMP-9 showed no utility in determining glioma disease status in a cohort of 111 GBM patients, and was not a clinically relevant prognostic marker of survival [54]. Ricci S et al. tested a series of 60 brain tumors (20 gliomas, 20 meningiomas and 20 metastases) and 25 controls and showed that MMP-9 is significantly increased in the sera from patients with CNS tumors compared to healthy controls [55]. Again, this study had a very small number of patients of each tumor type to allow solid conclusions.

Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) is a glycoprotein that is ubiquitously expressed in numerous human cells and tissues [56], with a wide spectrum of functions. Crocker data from 36 newly diagnosed GBM patients and five healthy controls showed that, in glioblastoma patients, a higher serum TIMP-1 level at presentation predicts shorter survival, and that TIMP-1 levels in the glioblastoma patients are significantly higher than in normal controls [57], suggesting that serum TIMP-1 levels are an independent predictor of survival. Aaberg-Jessen et al. in a cohort of 20 GBM patients did not find significant differences between plasma TIMP-1 levels and healthy control subjects [58]. They did not find any correlation between tissue levels and plasma levels, concluding that scoring TIMP-1 immunoreactivity is a better choice than measuring the plasma levels.

3.2. Biomarkers Related to Immune System

Immunologic factors have long been hypothesized to play a role in glioma risk, and secretion of immunosuppressant molecules by high-grade gliomas is well documented [59]. Some epidemiological studies have reported that adults with glioma are 1.5 to 4-fold less likely than controls to report a variety of allergies [60], supporting an inverse association between self-reported history of allergies and the risk of glioma. Indeed, a meta-analysis showed that glioma risk was reduced by 39% in people with a history of allergies compared with people with no history of allergies [61].

IgE is the class of antibody most responsible for atopic allergic diseases [62] and is a CD23 ligand [63]. An antitumor role for IgE has been proposed for solid tumors [64,65]. Wrensch et al. studied the serum IgE levels in a series of 115 GBM patients, showing that those with elevated levels had nine months longer survival than those with normal or borderline IgE levels [66]. Lin Yi et al. compared plasma IgE levels between different grades of gliomas in a series of 252 patients and 25 healthy controls and found that plasma IgE levels in low-grade glioma patients were lower than in high-grade glioma patients. No significant differences were found between grade III astrocytomas and GBM. For patients whose IgE increased significantly (increase > 100 ng/mL), the survival time was significantly longer than those patients whose IgE level did not increase significantly (127.5 ± 13.4 weeks vs. 62.3 ± 12.4 weeks). They found an increase of IgE levels in 24 patients after tumor treatment [65]. Calboli et al. analyzed data collected from four independent cohorts comprising 169 glioma cases and 520 controls and did not observe a statistically significant association between total IgE level and risk of glioma. A more careful examination revealed a lower risk for “borderline elevated” total IgE levels (25–100 kU/L) than with “elevated” total IgE levels (>100 kU/L), compared with clinically normal total IgE levels (<25 kU/L) [67]. Schlehofer B et al. in a case-control study found that the risk of glioma was inversely related to allergic sensitization and concluded that individuals with allergic sensitization are at reduced risk of developing glioma [68]. Schwartzbaum et al. conducted the largest study concerning the levels of IgE and the glioma risk and found that elevated levels of prediagnostic allergen-specific IgE and total IgE are associated with reduced risk of both glioblastoma and glioma, but the association between testing positive for allergen-specific IgE and decreased tumor risk was restricted to the female gender [44].

Despite some findings from most studies are conflicting, and more data should be obtained, it seems that there is an important correlation between IgE and GBM.

CD14 is an important component of the innate immune Toll-like receptor system [59] and CD23 is a mediator of the allergic response, exhibits proinflammatory properties and can function to enhance antigen presentation of IgE antigen complexes [59] and regulate IgE production [69]. In a case-control

study of 1078 cases for CD14, 1064 cases for CD23 and 736 controls, comprised 57 GBM patients among other malignant brain tumors, CD14 accumulated in GBM but not among the low-grade astrocytomas, in contrast to sCD23, which means serum levels was lower for glioma cases than controls [59]. Some reports suggest that elevated CD23, either on neoplastic cell surfaces or as a soluble form, is a useful marker in either diagnosis or prognosis of disease [63] and sCD23 levels could be used as a marker of glioma risk.

3.3. Biomarkers Related to Inflammation

Chronic inflammation increases the risk of developing some types of cancer and the inflammatory cell-driven microenvironment might lead to tumor initiation and promotion [10]. Several inflammatory markers were associated with the presence or progression of glioblastoma, and lymphocytic infiltration is a common finding.

Lopes M et al. studied the influence of neutrophil–lymphocyte ratio (NLR) in prognosis in a series of 140 GBM patients. They did not find NLR to be an independent poor prognostic factor in the total cohort, but in the subgroup of patients who completed the Stupp protocol, a preoperative NLR > 7 correlated with shorter overall survival [70]. These results do not corroborate the previous findings of Bambury et al. and of Han et al. showing a significant correlation between NLR > 4 and a shorter overall survival [71,72].

IL-6 amplification is also involved in angiogenesis and tumor progression, and although it was associated with shorter survival in glioblastoma patients [73], Reynés et al., in a series of 40 GBM patients and 60 healthy controls, found that all inflammation markers studied, including IL-6, were significantly elevated in glioblastoma patients compared with controls [10]. Shan Y et al. studied a series of 16 healthy controls and 86 glioma patients, 43 of which were GBM [74], and found IL-6 in the serum of glioma patients increased as compared to the control group, and this increase was related to the grade of glioma and reduced markedly after surgery. These decreases were closely related to the prognosis of glioma patients and could be used for prediction of clinical prognosis [74].

Amyloid A1 (SAA1) is a sensitive acute phase reactant primarily produced by the liver in response to acute inflammation and affects proliferation, migration and invasion of glioblastoma cell lines [75]. There is evidence that elevated serum levels of SAA correlate directly with poor prognosis in various systemic cancers such as lung, breast, melanoma, gastric, prostate, esophageal, endometrial and renal [75].

Knebel et al. in a series of 148 astrocytoma and 10 non-neoplastic patients showed that patients with astrocytic tumors have higher SAA serum concentrations than the non-neoplastic controls. However, they did not find statistically significant differences among the different astrocytoma grades [75]. The SAA produced by glioblastoma did not contribute to the increase in serum SAA, rather this high serum concentration is due to liver production as part as an inflammatory response to the tumor. They found no association between SAA serum levels and patient survival.

Alpha 2-Heremans-Schmid glycoprotein (AHSG) is a 64-kDa serum protein, a liver-derived circulating protein and is a ligand for tyrosine kinase receptors [76]. AHSG is a negative acute-phase protein. The serum AHSG concentration is decreased in individuals with infection, malignancies, liver diseases, malnutrition and end-stage renal disease [77]. It also has a link between obesity, fatty liver, insulin resistance, and metabolic syndromes [78].

There are two studies concerning AHSG and brain tumors. Ribom et al., in a 2003, paper showed an increase in CSF levels in patients with low-grade gliomas [79]. Petrik et al. first determined AHSG as a survival predictor in GBM and then, in an additional series of 72 GBM patients, validated its prognostic value. They demonstrated that low serum alpha 2-HS glycoprotein concentration correlates with increased tumor proliferation. However, reduced serum AHSG is not a specific biomarker of GBM. It is also low in patients with non-glioma brain pathologies and in patients with non-CNS tumors [80].

Heat shock 70-kDa protein is a heat shock protein (HSP), which is a group of phylogenetically conserved proteins found in all prokaryotic and eukaryotic cells [81]. Increased HSP70 antibody levels

have been found in the serum of lung cancer patients, indicating that it may be a biomarker for several tumor types [7]. Different patterns of HSP expression seem not to account for the differential response of these tumors to adjuvant cytotoxic therapy [82].

Glioblastoma patients have an increase serum concentration of HSP70 compared with normal controls but in the Elstner study, HSP70 was only reliable when associated with BMP2 and CXCL10 [7]. No additional evidence has been reported to support the association between HSP70 and glioma, making this protein a weak biomarker for prognosis and survival.

3.4. Biomarkers Related to Nutritional Status

The nutritional status has been studied in many cancers, being a prognostic factor. The association between nutritional status and inflammatory status may play a role in the development of glioblastoma and seems to be an important prognostic factor. The biomarkers described to be related to glioblastoma are albumin and IGFBP-2.

Serum albumin measures long-standing malnutrition and is also associated with systemic inflammation.

Borg et al. analyzed a series of 549 GBM patients with pre-operative albumin assayed. They showed significantly higher perioperative mortality (≤ 30 days post-operatively) in the hypoalbuminemia group, which also showed significantly lower conditional survival after 30 days. They could not find a definite reason but correlated indirectly to an inflammatory status mediated by cytokines TNF α and IL-6 [83].

Han et al. showed that patients with low serum albumin levels (<30 g/L) had a significantly shorter overall survival than those with levels in the normal range (median 6.0 vs. 15.0 months) and this is partial due to the nutritional status of patients but mostly to the inflammatory response and higher IGFBP-2 levels [72].

The prognostic nutritional index (PNI), which was calculated from the following formula: $10 \times \text{serum albumin (g/dL)} + 0.005 \times \text{total lymphocyte count (per-mm}^3\text{)}$, reflected the nutritional and immune status of patients with cancer [84].

The preoperative albumin-to-globulin ratio (AGR) could reflect both malnutrition and systemic inflammation in cancer patients. In Liu study, patients with low preoperative albumin-to-globulin ratio have a better prognosis and conclude that preoperative AGR was significantly associated with OS in GBM patients [85].

The possible mechanism by which PNI is involved with tumor prognosis may be that a low serum albumin level deteriorates the general nutritional status of the patients [84].

He et al. created a cumulative score using serum albumin plus plasma fibrinogen. Fibrinogen was shown to play important roles both in tumor cell proliferation, migration and angiogenesis and as regulator of inflammation in solid tumors. They conclude that higher pretreatment FA score was an independent prognostic factor of PFS and OS of patients with high-grade gliomas and was significantly associated with advanced age and higher tumor grade [86].

3.5. Biomarkers Related to Coagulation

Thrombin expression is involved in brain development, protection and regeneration. Besides its role in hemostasis, thrombin facilitates receptor-mediated inflammatory responses, apoptosis, cell proliferation/modulation and angiogenesis, and is involved in complex signaling pathways in glioblastoma [87].

Reynés G et al. found that prothrombin fragments 1 + 2 and endogen thrombin generation levels were significantly higher in glioblastoma patients than in controls [10]. No further studies associated serum expression of coagulation markers and glioblastoma, even though these factors have a preponderant role in inflammation and gliomagenesis.

3.6. Circulating Tumor DNA

Circulating tumor DNA (ctDNA) has been the subject of investigations in the last few years. Despite the technical challenges and the very low concentrations of nucleic acid in biofluids, the information obtained is of paramount importance [88]. In normal individual and in early-stage cancers the ctDNA concentrations are very low, but these levels significantly increase with tumor progression and there is growing evidence that ctDNA concentrations are related to tumor burden, cellular turnover, cancer stage, and therapy response [89]. ctDNA could be obtained regularly over time, reflecting the real composition, the tumoral heterogeneity and the evolution of the tumor throughout time [90]. ctDNA allows us to overcome some difficulties of the magnetic resonance imaging (MRI) in the follow-up of the patients. Treatment effects (pseudoprogression), progressive disease and radiation necrosis are not always easy to distinguish in advanced brain imaging modalities or metabolic imaging such as PET scan [89].

Piccioni et al. studied 665 blood samples from 419 consecutive patients with primary brain tumors. Somatic alterations were detected in 302 samples and in 55% of GBM patients. One-quarter of samples had a ctDNA alteration detected that suggested eligibility for an off-label targeted therapy regimen and almost half of patients had a ctDNA alteration detected that suggested eligibility for a targeted therapy clinical trial [91]. Saenz-Antonanzas et al. concluded that ctDNA has great potential to identify biomarkers for diagnosis, prognosis, and therapeutic response and allow genotype-directed therapies and personalized clinical management of glioblastoma patients [90].

4. Conclusions

The pivotal criterion with regard to the potential clinical value of a candidate cancer biomarker is the consistency and strength of the association between the biomarker and the outcome or disease of interest, and the extent to which it is an improvement on either adding to or replacing established tools [5]. Of the various putative GBM biomarkers, YKL-40 emerges as particularly promising in various studies, suggesting it may be useful as a prognostic predictor and may have a role in screening and monitoring of GBM patients.

Although a significant activation of inflammation, angiogenesis and coagulation processes have been demonstrated in patients with glioblastoma, none of the reported circulating markers could be associated with clinical outcome.

Single molecular markers are mostly insufficient to follow the dynamics of diseases such as cancer and will be replaced by multiple marker profiles. When in conjunction, the serum concentrations of certain proteins may indicate the presence of a glioblastoma with high sensitivity and specificity.

Despite these findings, none of these proteins alone was sufficiently specific and sensitive to serve as a diagnostic marker. It is important to note that none of these markers have been implemented in the clinical routine and so do not have clinical value. We can speculate that this lack of consistent results is due to the disease and patient heterogeneity. Another explanation is the fact that some studies have a reduced number of patients, lack of standardization in the sample collection, different quantification methods and lack of results validation. Most studies are small retrospective series that do not allow robust statistical conclusions. The methodology used varies depending on the biomarker studied, with no uniform criteria allowing homogenization of the results. The cut-offs used are different in various studies. The follow-up period is short. Many of the biomarkers were only collected preoperatively, with no longitudinal assessment over the course of the disease. Most markers are involved in various physiological and pathological processes and are not specific to glioblastomas, contradicting the definition of biomarker. The concomitance of other pathologies with increased expression of the same biomarkers was not excluded in most studies. Another important limitation is that a subset of patients with GBM have normal serum levels of these markers.

In the complex context of gliomagenesis, progression, and regrowth, with a multifactorial network involving vascular proliferation, cell growth, inflammatory, immunological, coagulation and nutritional factors, trying to address only one factor that could function as a biomarker of diagnosis, prognosis or

disease status could be impossible and only a set of biomarkers can actually give appropriate and more accurate information. However, at the moment, this is not possible given the length of the process and the costs involved, which makes it inappropriate for each patient per se.

We conclude that although these markers may be useful in identifying gliomas, they lack enough acuity to avoid biopsy and tissue analysis. This is the reason why there is currently no true blood biomarker specific to glioblastomas. In the future, a multi-biomarker panel signature may prove to be a very useful tool in glioma subtypes diagnosis, improving prognosis information and treatment response prediction. Single-cell analyses, ctDNA or circulating tumor cells analyses are promising technologies that may improve diagnostic accuracy and quality of follow-up in GBM patients. In this perspective, it is our conviction that research must be consolidated by prospective studies targeting routine, low-cost, disease-specific biomarkers allowing early diagnosis, better prognosis stratification and response prediction. Additionally, the characterization of a panel of genetic alterations in glioblastoma specimens that match peripheral blood markers, may increase the sensitivity of the screening biomarkers. Our main effort at this point is focused on ctDNA, namely in identifying genomic alterations that may allow a more efficient follow-up and possible targeted therapies, matching off-label and clinical trials agents.

Author Contributions: Conceptualization, methodology, writing—original draft preparation, P.L.; writing—review and editing, P.L., B.C., R.V. and B.M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Louis, D.N.; Ohgaki, H.; Wiestler, O.D.; Cavenee, W.K.; Burger, P.C.; Jouvet, A.; Scheithauer, B.W.; Kleihues, P. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* **2007**, *114*, 97–109. [CrossRef]
2. Riemenschneider, M.J.; Jeuken, J.W.; Wesseling, P.; Reifenberger, G. Molecular diagnostics of gliomas: State of the art. *Acta Neuropathol.* **2010**, *120*, 567–584. [CrossRef] [PubMed]
3. Johansen, J.S.; Jensen, B.V.; Roslind, A.; Nielsen, D.; Price, P.A. Serum YKL-40, a new prognostic biomarker in cancer patients? *Cancer Epidemiol Biomark. Prev.* **2006**, *15*, 194–202. [CrossRef] [PubMed]
4. McDonald, K.L.; Aw, G.; Kleihues, P. Role of Biomarkers in the Clinical Management of Glioblastomas: What are the Barriers and How Can We Overcome Them? *Front. Neurol.* **2012**, *3*, 188. [CrossRef] [PubMed]
5. Schultz, N.A.; Johansen, J.S. YKL-40-A Protein in the Field of Translational Medicine: A Role as a Biomarker in Cancer Patients? *Cancers* **2010**, *2*, 1453–1491. [CrossRef]
6. Tanwar, M.K.; Gilbert, M.R.; Holland, E.C. Gene expression microarray analysis reveals YKL-40 to be a potential serum marker for malignant character in human glioma. *Cancer Res.* **2002**, *62*, 4364–4368.
7. Elstner, A.; Stockhammer, F.; Nguyen-Dobinsky, T.N.; Nguyen, Q.L.; Pilgermann, I.; Gill, A.; Guhr, A.; Zhang, T.; von Eckardstein, K.; Picht, T.; et al. Identification of diagnostic serum protein profiles of glioblastoma patients. *J. Neurooncol.* **2011**, *102*, 71–80. [CrossRef]
8. Sampath, P.; Weaver, C.E.; Sungarian, A.; Cortez, S.; Alderson, L.; Stopa, E.G. Cerebrospinal fluid (vascular endothelial growth factor) and serologic (recoverin) tumor markers for malignant glioma. *Cancer Control* **2004**, *11*, 174–180. [CrossRef]
9. Franco-Hernandez, C.; Martinez-Glez, V.; Rey, J.A. Biology molecular of glioblastomas. *Neurocir. (Astur)* **2007**, *18*, 373–382. Available online: <https://pubmed.ncbi.nlm.nih.gov/18008011/> (accessed on 8 August 2020). [CrossRef]
10. Reynes, G.; Vila, V.; Martin, M.; Parada, A.; Fleitas, T.; Reganon, E.; Martinez-Sales, V. Circulating markers of angiogenesis, inflammation, and coagulation in patients with glioblastoma. *J. Neurooncol.* **2011**, *102*, 35–41. [CrossRef]
11. Salven, P.; Manpaa, H.; Orpana, A.; Alitalo, K.; Joensuu, H. Serum vascular endothelial growth factor is often elevated in disseminated cancer. *Clin. Cancer Res.* **1997**, *3*, 647–651. [PubMed]

12. Stockhammer, G.; Obwegeser, A.; Kostron, H.; Schumacher, P.; Muigg, A.; Felber, S.; Maier, H.; Slavc, I.; Gunsilius, E.; Gastl, G. Vascular endothelial growth factor (VEGF) is elevated in brain tumor cysts and correlates with tumor progression. *Acta Neuropathol.* **2000**, *100*, 101–105. [[CrossRef](#)] [[PubMed](#)]
13. Fine, H.A.; Figg, W.D.; Jaeckle, K.; Wen, P.Y.; Kyritsis, A.P.; Loeffler, J.S.; Levin, V.A.; Black, P.M.; Kaplan, R.; Pluda, J.M.; et al. Phase II trial of the antiangiogenic agent thalidomide in patients with recurrent high-grade gliomas. *J. Clin. Oncol.* **2000**, *18*, 708–715. [[CrossRef](#)] [[PubMed](#)]
14. Rafat, N.; Beck, G.; Schulte, J.; Tuettenberg, J.; Vajkoczy, P. Circulating endothelial progenitor cells in malignant gliomas. *J. Neurosurg.* **2010**, *112*, 43–49. [[CrossRef](#)]
15. Bojesen, S.E.; Johansen, J.S.; Nordestgaard, B.G. Plasma YKL-40 levels in healthy subjects from the general population. *Clin. Chim. Acta* **2011**, *412*, 709–712. [[CrossRef](#)]
16. Zhang, W.; Kawanishi, M.; Miyake, K.; Kagawa, M.; Kawai, N.; Murao, K.; Nishiyama, A.; Fei, Z.; Zhang, X.; Tamiya, T. Association between YKL-40 and adult primary astrocytoma. *Cancer* **2010**, *116*, 2688–2697. [[CrossRef](#)]
17. Nigro, J.M.; Misra, A.; Zhang, L.; Smirnov, I.; Colman, H.; Griffin, C.; Ozburn, N.; Chen, M.; Pan, E.; Koul, D.; et al. Integrated array-comparative genomic hybridization and expression array profiles identify clinically relevant molecular subtypes of glioblastoma. *Cancer Res.* **2005**, *65*, 1678–1686. [[CrossRef](#)]
18. Hormigo, A.; Gu, B.; Karimi, S.; Riedel, E.; Panageas, K.S.; Edgar, M.A.; Tanwar, M.K.; Rao, J.S.; Fleisher, M.; DeAngelis, L.M.; et al. YKL-40 and matrix metalloproteinase-9 as potential serum biomarkers for patients with high-grade gliomas. *Clin. Cancer Res.* **2006**, *12*, 5698–5704. [[CrossRef](#)]
19. Bernardi, D.; Padoan, A.; Ballin, A.; Sartori, M.; Manara, R.; Scienza, R.; Plebani, M.; Della Puppa, A. Serum YKL-40 following resection for cerebral glioblastoma. *J. Neurooncol.* **2012**, *107*, 299–305. [[CrossRef](#)]
20. van Linde, M.E.; van der Mij, J.C.; Pham, T.V.; Knol, J.C.; Wedekind, L.E.; Hovinga, K.E.; Aliaga, E.S.; Buter, J.; Jimenez, C.R.; Reijneveld, J.C.; et al. Evaluation of potential circulating biomarkers for prediction of response to chemoradiation in patients with glioblastoma. *J. Neurooncol.* **2016**, *129*, 221–230. [[CrossRef](#)]
21. Johansen, J.S.; Schultz, N.A.; Jensen, B.V. Plasma YKL-40: A potential new cancer biomarker? *Future Oncol.* **2009**, *5*, 1065–1082. [[CrossRef](#)] [[PubMed](#)]
22. Huang, J.; Hu, J.; Bian, X.; Chen, K.; Gong, W.; Dunlop, N.M.; Howard, O.M.; Wang, J.M. Transactivation of the epidermal growth factor receptor by formylpeptide receptor exacerbates the malignant behavior of human glioblastoma cells. *Cancer Res.* **2007**, *67*, 5906–5913. [[CrossRef](#)]
23. Costa, B.M.; Viana-Pereira, M.; Fernandes, R.; Costa, S.; Linhares, P.; Vaz, R.; Pinheiro, C.; Lima, J.; Soares, P.; Silva, A.; et al. Impact of EGFR genetic variants on glioma risk and patient outcome. *Cancer Epidemiol. Biomark. Prev.* **2011**, *20*, 2610–2617. [[CrossRef](#)]
24. Zawrocki, A.; Biernat, W. Epidermal growth factor receptor in glioblastoma. *Folia. Neuropathol.* **2005**, *43*, 123–132. [[PubMed](#)]
25. Choi, Y.; Song, Y.J.; Lee, H.S.; Hur, W.J.; Sung, K.H.; Kim, K.U.; Choi, S.S.; Kim, S.J.; Kim, D.C. Epidermal growth factor receptor is related to poor survival in glioblastomas: Single-institution experience. *Yonsei Med. J.* **2013**, *54*, 101–107. [[CrossRef](#)] [[PubMed](#)]
26. Quaranta, M.; Divella, R.; Daniele, A.; Di Tardo, S.; Venneri, M.T.; Lolli, I.; Troccoli, G. Epidermal growth factor receptor serum levels and prognostic value in malignant gliomas. *Tumori* **2007**, *93*, 275–280. [[CrossRef](#)] [[PubMed](#)]
27. Wachsberger, P.R.; Lawrence, R.Y.; Liu, Y.; Rice, B.; Daskalakis, C.; Dicker, A.P. Epidermal growth factor receptor mutation status and rad51 determine the response of glioblastoma to multimodality therapy with cetuximab, temozolomide, and radiation. *Front. Oncol.* **2013**, *3*, 13. [[CrossRef](#)] [[PubMed](#)]
28. Barker, F.G., 2nd; Simmons, M.L.; Chang, S.M.; Prados, M.D.; Larson, D.A.; Sneed, P.K.; Wara, W.M.; Berger, M.S.; Chen, P.; Israel, M.A.; et al. EGFR overexpression and radiation response in glioblastoma multiforme. *Int. J. Radiat. Oncol. Biol. Phys.* **2001**, *51*, 410–418. [[CrossRef](#)]
29. Husain, H.; Savage, W.; Grossman, S.A.; Ye, X.; Burger, P.C.; Everett, A.; Bettegowda, C.; Diaz, L.A., Jr.; Blair, C.; Romans, K.E.; et al. Pre- and post-operative plasma glial fibrillary acidic protein levels in patients with newly diagnosed gliomas. *J. Neurooncol.* **2012**, *109*, 123–127. [[CrossRef](#)] [[PubMed](#)]
30. Brommelund, T.; Rosengren, L.; Fridlund, S.; Hennig, R.; Isaksen, V. Serum levels of glial fibrillary acidic protein correlate to tumour volume of high-grade gliomas. *Acta Neurol. Scand* **2007**, *116*, 380–384. [[CrossRef](#)]

31. Jung, C.S.; Foerch, C.; Schanzer, A.; Heck, A.; Plate, K.H.; Seifert, V.; Steinmetz, H.; Raabe, A.; Sitzer, M. Serum GFAP is a diagnostic marker for glioblastoma multiforme. *Brain* **2007**, *130*, 3336–3341. [[CrossRef](#)] [[PubMed](#)]
32. Ilhan-Mutlu, A.; Wagner, L.; Widhalm, G.; Wohrer, A.; Bartsch, S.; Czech, T.; Heinzl, H.; Leutmezer, F.; Prayer, D.; Marosi, C.; et al. Exploratory investigation of eight circulating plasma markers in brain tumor patients. *Neurosurg. Rev.* **2013**, *36*, 45–55, discussion 55–46. [[CrossRef](#)]
33. Vietheer, J.M.; Rieger, J.; Wagner, M.; Senft, C.; Tichy, J.; Foerch, C. Serum concentrations of glial fibrillary acidic protein (GFAP) do not indicate tumor recurrence in patients with glioblastoma. *J. Neurooncol.* **2017**, *135*, 193–199. [[CrossRef](#)] [[PubMed](#)]
34. Atai, N.A.; Bansal, M.; Lo, C.; Bosman, J.; Tigchelaar, W.; Bosch, K.S.; Jonker, A.; De Witt Hamer, P.C.; Troost, D.; McCulloch, C.A.; et al. Osteopontin is up-regulated and associated with neutrophil and macrophage infiltration in glioblastoma. *Immunology* **2011**, *132*, 39–48. [[CrossRef](#)] [[PubMed](#)]
35. Jang, T.; Savarese, T.; Low, H.P.; Kim, S.; Vogel, H.; Lapointe, D.; Duong, T.; Litofsky, N.S.; Weimann, J.M.; Ross, A.H.; et al. Osteopontin expression in intratumoral astrocytes marks tumor progression in gliomas induced by prenatal exposure to N-ethyl-N-nitrosourea. *Am. J. Pathol.* **2006**, *168*, 1676–1685. [[CrossRef](#)]
36. Yan, W.; Qian, C.; Zhao, P.; Zhang, J.; Shi, L.; Qian, J.; Liu, N.; Fu, Z.; Kang, C.; Pu, P.; et al. Expression pattern of osteopontin splice variants and its functions on cell apoptosis and invasion in glioma cells. *Neuro. Oncol.* **2010**, *12*, 765–775. [[CrossRef](#)]
37. Matusan-Ilijas, K.; Behrem, S.; Jonjic, N.; Zarkovic, K.; Lucin, K. Osteopontin expression correlates with angiogenesis and survival in malignant astrocytoma. *Pathol. Oncol. Res.* **2008**, *14*, 293–298. [[CrossRef](#)]
38. Sreekanthreddy, P.; Srinivasan, H.; Kumar, D.M.; Nijaguna, M.B.; Sridevi, S.; Vrinda, M.; Arivazhagan, A.; Balasubramaniam, A.; Hegde, A.S.; Chandramouli, B.A.; et al. Identification of potential serum biomarkers of glioblastoma: Serum osteopontin levels correlate with poor prognosis. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 1409–1422. [[CrossRef](#)]
39. Thirkill, C.E.; Roth, A.M.; Keltner, J.L. Cancer-associated retinopathy. *Arch. Ophthalmol.* **1987**, *105*, 372–375. [[CrossRef](#)]
40. Manley, P.; Li, X.; Turner, C.; Chi, S.; Zimmerman, M.A.; Chordas, C.; Gordon, A.; Baker, A.; Ullrich, N.J.; Goumnerova, L.; et al. A prospective, blinded analysis of A-PROTEIN (recoverin or CAR protein) levels in pediatric patients with central nervous system tumors. *Pediatr. Blood Cancer* **2009**, *53*, 343–347. [[CrossRef](#)]
41. Fukuda, M.E.; Iwade, Y.; Machida, T.; Hiwasa, T.; Nimura, Y.; Nagai, Y.; Takiguchi, M.; Tanzawa, H.; Yamaura, A.; Seki, N. Cathepsin D is a potential serum marker for poor prognosis in glioma patients. *Cancer Res.* **2005**, *65*, 5190–5194. [[CrossRef](#)]
42. Whitaker, J.N.; Terry, L.C.; Whetsell, W.O., Jr. Immunocytochemical localization of cathepsin D in rat neural tissue. *Brain Res.* **1981**, *216*, 109–124. [[CrossRef](#)]
43. Sun, S.; Wong, T.S.; Zhang, X.Q.; Pu, J.K.; Lee, N.P.; Day, P.J.; Ng, G.K.; Lui, W.M.; Leung, G.K. Protein alterations associated with temozolomide resistance in subclones of human glioblastoma cell lines. *J. Neurooncol.* **2012**, *107*, 89–100. [[CrossRef](#)] [[PubMed](#)]
44. Schwartzbaum, J.; Ding, B.; Johannesen, T.B.; Osnes, L.T.; Karavodin, L.; Ahlbom, A.; Feychting, M.; Grimsrud, T.K. Association between prediagnostic IgE levels and risk of glioma. *J. Natl. Cancer Inst.* **2012**, *104*, 1251–1259. [[CrossRef](#)] [[PubMed](#)]
45. Loh, J.K.; Lieu, A.S.; Su, Y.F.; Cheng, C.Y.; Tsai, T.H.; Lin, C.L.; Lee, K.S.; Hwang, S.L.; Kwan, A.L.; Wang, C.J.; et al. The alteration of plasma TGF-beta1 levels in patients with brain tumors after tumor removal. *Kaohsiung J. Med. Sci.* **2012**, *28*, 316–321. [[CrossRef](#)]
46. Chattopadhyay, N.; Jacob, T.; Hansen, F.; Godbole, M.M.; Brown, E.M. Transforming growth factor beta receptor family ligands inhibit hepatocyte growth factor synthesis and secretion from astrocytoma cells. *Brain Res. Mol. Brain Res.* **2004**, *121*, 146–150. [[CrossRef](#)]
47. Liu, C.; Tian, G.; Tu, Y.; Fu, J.; Lan, C.; Wu, N. Expression pattern and clinical prognostic relevance of bone morphogenetic protein-2 in human gliomas. *Jpn. J. Clin. Oncol.* **2009**, *39*, 625–631. [[CrossRef](#)]
48. Nijaguna, M.B.; Patil, V.; Hegde, A.S.; Chandramouli, B.A.; Arivazhagan, A.; Santosh, V.; Somasundaram, K. An Eighteen Serum Cytokine Signature for Discriminating Glioma from Normal Healthy Individuals. *PLoS ONE* **2015**, *10*, e0137524. [[CrossRef](#)]

49. Samaras, V.; Piperi, C.; Levidou, G.; Zisakis, A.; Kavantzias, N.; Themistocleous, M.S.; Boviatsis, E.I.; Barbatis, C.; Lea, R.W.; Kalofoutis, A.; et al. Analysis of interleukin (IL)-8 expression in human astrocytomas: Associations with IL-6, cyclooxygenase-2, vascular endothelial growth factor, and microvessel morphometry. *Hum. Immunol.* **2009**, *70*, 391–397. [\[CrossRef\]](#)
50. Popescu, I.D.; Codrici, E.; Albuiescu, L.; Mihai, S.; Enciu, A.M.; Albuiescu, R.; Tanase, C.P. Potential serum biomarkers for glioblastoma diagnostic assessed by proteomic approaches. *Proteome Sci.* **2014**, *12*, 47. [\[CrossRef\]](#)
51. Djonov, V.; Cresto, N.; Aebbersold, D.M.; Burri, P.H.; Altermatt, H.J.; Hristic, M.; Berclaz, G.; Ziemiecki, A.; Andres, A.C. Tumor cell specific expression of MMP-2 correlates with tumor vascularisation in breast cancer. *Int. J. Oncol.* **2002**, *21*, 25–30. [\[CrossRef\]](#)
52. Lucio-Eterovic, A.K.; Piao, Y.; de Groot, J.F. Mediators of glioblastoma resistance and invasion during antivascular endothelial growth factor therapy. *Clin. Cancer Res.* **2009**, *15*, 4589–4599. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Raithatha, S.A.; Muzik, H.; Muzik, H.; Rewcastle, N.B.; Johnston, R.N.; Edwards, D.R.; Forsyth, P.A. Localization of gelatinase-A and gelatinase-B mRNA and protein in human gliomas. *Neuro. Oncol.* **2000**, *2*, 145–150. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Iwamoto, F.M.; Hottinger, A.F.; Karimi, S.; Riedel, E.; Dantis, J.; Jahdi, M.; Panageas, K.S.; Lassman, A.B.; Abrey, L.E.; Fleisher, M.; et al. Longitudinal prospective study of matrix metalloproteinase-9 as a serum marker in gliomas. *J. Neurooncol.* **2011**, *105*, 607–612. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Ricci, S.; Guadagno, E.; Bruzzese, D.; Del Basso De Caro, M.; Peca, C.; Sgulo, F.G.; Maiuri, F.; Di Carlo, A. Evaluation of matrix metalloproteinase type IV-collagenases in serum of patients with tumors of the central nervous system. *J. Neurooncol.* **2017**, *131*, 223–232. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Egea, V.; Zahler, S.; Rieth, N.; Neth, P.; Popp, T.; Kehe, K.; Jochum, M.; Ries, C. Tissue inhibitor of metalloproteinase-1 (TIMP-1) regulates mesenchymal stem cells through let-7f microRNA and Wnt/beta-catenin signaling. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E309–E316. [\[CrossRef\]](#)
57. Crocker, M.; Ashley, S.; Giddings, I.; Petrik, V.; Hardcastle, A.; Aherne, W.; Pearson, A.; Bell, B.A.; Zacharoulis, S.; Papadopoulos, M.C. Serum angiogenic profile of patients with glioblastoma identifies distinct tumor subtypes and shows that TIMP-1 is a prognostic factor. *Neuro. Oncol.* **2011**, *13*, 99–108. [\[CrossRef\]](#)
58. Aaberg-Jessen, C.; Halle, B.; Jensen, S.S.; Muller, S.; Romer, U.M.; Pedersen, C.B.; Brunner, N.; Kristensen, B.W. Comparative studies of TIMP-1 immunohistochemistry, TIMP-1 FISH analysis and plasma TIMP-1 in glioblastoma patients. *J. Neurooncol.* **2016**, *130*, 439–448. [\[CrossRef\]](#)
59. Zhou, M.; Wiemels, J.L.; Bracci, P.M.; Wrensch, M.R.; McCoy, L.S.; Rice, T.; Sison, J.D.; Patoka, J.S.; Wiencke, J.K. Circulating levels of the innate and humoral immune regulators CD14 and CD23 are associated with adult glioma. *Cancer Res.* **2010**, *70*, 7534–7542. [\[CrossRef\]](#)
60. Wiemels, J.L.; Wilson, D.; Patil, C.; Patoka, J.; McCoy, L.; Rice, T.; Schwartzbaum, J.; Heimberger, A.; Sampson, J.H.; Chang, S.; et al. IgE, allergy, and risk of glioma: Update from the San Francisco Bay Area Adult Glioma Study in the temozolomide era. *Int. J. Cancer* **2009**, *125*, 680–687. [\[CrossRef\]](#)
61. Linos, E.; Raine, T.; Alonso, A.; Michaud, D. Atopy and risk of brain tumors: A meta-analysis. *J. Natl. Cancer Inst.* **2007**, *99*, 1544–1550. [\[CrossRef\]](#)
62. Wiemels, J.L.; Wiencke, J.K.; Patoka, J.; Moghadassi, M.; Chew, T.; McMillan, A.; Miiike, R.; Barger, G.; Wrensch, M. Reduced immunoglobulin E and allergy among adults with glioma compared with controls. *Cancer Res.* **2004**, *64*, 8468–8473. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Acharya, M.; Borland, G.; Edkins, A.L.; Maclellan, L.M.; Matheson, J.; Ozanne, B.W.; Cushley, W. CD23/FcepsilonRII: Molecular multi-tasking. *Clin. Exp. Immunol.* **2010**, *162*, 12–23. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Fu, S.L.; Pierre, J.; Smith-Norowitz, T.A.; Hagler, M.; Bowne, W.; Pincus, M.R.; Mueller, C.M.; Zenilman, M.E.; Bluth, M.H. Immunoglobulin E antibodies from pancreatic cancer patients mediate antibody-dependent cell-mediated cytotoxicity against pancreatic cancer cells. *Clin. Exp. Immunol.* **2008**, *153*, 401–409. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Lin, Y.; Jin, Q.; Zhang, G.Z.; Wang, Y.J.; Jiang, T.; Wu, A.H.; Wang, J.F.; Qiu, X.G. Increase of plasma IgE during treatment correlates with better outcome of patients with glioblastoma. *Chin. Med. J. (Engl.)* **2011**, *124*, 3042–3048. [\[PubMed\]](#)

66. Wrensch, M.; Wiencke, J.K.; Wiemels, J.; Mücke, R.; Patoka, J.; Moghadassi, M.; McMillan, A.; Kelsey, K.T.; Aldape, K.; Lamborn, K.R.; et al. Serum IgE, tumor epidermal growth factor receptor expression, and inherited polymorphisms associated with glioma survival. *Cancer Res.* **2006**, *66*, 4531–4541. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Calboli, F.C.; Cox, D.G.; Buring, J.E.; Gaziano, J.M.; Ma, J.; Stampfer, M.; Willett, W.C.; Tworoger, S.S.; Hunter, D.J.; Camargo, C.A., Jr.; et al. Prediagnostic plasma IgE levels and risk of adult glioma in four prospective cohort studies. *J. Natl. Cancer Inst.* **2011**, *103*, 1588–1595. [\[CrossRef\]](#)
68. Schlehofer, B.; Siegmund, B.; Linseisen, J.; Schuz, J.; Rohrmann, S.; Becker, S.; Michaud, D.; Melin, B.; Bas Bueno-de-Mesquita, H.; Peeters, P.H.; et al. Primary brain tumours and specific serum immunoglobulin E: A case-control study nested in the European Prospective Investigation into Cancer and Nutrition cohort. *Allergy* **2011**, *66*, 1434–1441. [\[CrossRef\]](#)
69. Olteanu, H.; Fenske, T.S.; Harrington, A.M.; Szabo, A.; He, P.; Kroft, S.H. CD23 expression in follicular lymphoma: Clinicopathologic correlations. *Am. J. Clin. Pathol.* **2011**, *135*, 46–53. [\[CrossRef\]](#) [\[PubMed\]](#)
70. Lopes, M.; Carvalho, B.; Vaz, R.; Linhares, P. Influence of neutrophil-lymphocyte ratio in prognosis of glioblastoma multiforme. *J. Neurooncol.* **2018**, *136*, 173–180. [\[CrossRef\]](#)
71. Bambury, R.M.; Teo, M.Y.; Power, D.G.; Yusuf, A.; Murray, S.; Battley, J.E.; Drake, C.; O'Dea, P.; Bermingham, N.; Keohane, C.; et al. The association of pre-treatment neutrophil to lymphocyte ratio with overall survival in patients with glioblastoma multiforme. *J. Neurooncol.* **2013**, *114*, 149–154. [\[CrossRef\]](#)
72. Han, S.; Meng, L.; Han, S.; Wang, Y.; Wu, A. Plasma IGFBP-2 levels after postoperative combined radiotherapy and chemotherapy predict prognosis in elderly glioblastoma patients. *Plos ONE* **2014**, *9*, e93791. [\[CrossRef\]](#) [\[PubMed\]](#)
73. Tchirkov, A.; Khalil, T.; Chautard, E.; Mokhtari, K.; Veronese, L.; Irthum, B.; Vago, P.; Kemeny, J.L.; Verrelle, P. Interleukin-6 gene amplification and shortened survival in glioblastoma patients. *Br. J. Cancer* **2007**, *96*, 474–476. [\[CrossRef\]](#) [\[PubMed\]](#)
74. Shan, Y.; He, X.; Song, W.; Han, D.; Niu, J.; Wang, J. Role of IL-6 in the invasiveness and prognosis of glioma. *Int. J. Clin. Exp. Med.* **2015**, *8*, 9114–9120. [\[PubMed\]](#)
75. Knebel, F.H.; Albuquerque, R.C.; Massaro, R.R.; Maria-Engler, S.S.; Campa, A. Dual effect of serum amyloid A on the invasiveness of glioma cells. *Mediat. Inflamm.* **2013**, *2013*, 509089. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Jenkins, N.T.; McKenzie, J.A.; Hagberg, J.M.; Witkowski, S. Plasma fetuin-A concentrations in young and older high- and low-active men. *Metabolism* **2011**, *60*, 265–271. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Sato, H.; Kazama, J.J.; Wada, Y.; Kuroda, T.; Narita, I.; Gejyo, F.; Gao, P.; Yamashita, H. Decreased levels of circulating alpha2-Heremans-Schmid glycoprotein/Fetuin-A (AHSG) in patients with rheumatoid arthritis. *Intern. Med.* **2007**, *46*, 1685–1691. [\[CrossRef\]](#)
78. Reinehr, T.; Roth, C.L. Fetuin-A and its relation to metabolic syndrome and fatty liver disease in obese children before and after weight loss. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 4479–4485. [\[CrossRef\]](#)
79. Ribom, D.; Westman-Brinkmalm, A.; Smits, A.; Davidsson, P. Elevated levels of alpha-2-Heremans-Schmid glycoprotein in CSF of patients with low-grade gliomas. *Tumour. Biol.* **2003**, *24*, 94–99. [\[CrossRef\]](#)
80. Petrik, V.; Saadoun, S.; Loosemore, A.; Hobbs, J.; Opstad, K.S.; Sheldon, J.; Tarelli, E.; Howe, F.A.; Bell, B.A.; Papadopoulos, M.C. Serum alpha 2-HS glycoprotein predicts survival in patients with glioblastoma. *Clin. Chem* **2008**, *54*, 713–722. [\[CrossRef\]](#)
81. Mansilla, M.J.; Montalban, X.; Espejo, C. Heat shock protein 70: Roles in multiple sclerosis. *Mol. Med.* **2012**, *18*, 1018–1028. [\[CrossRef\]](#)
82. Strik, H.M.; Weller, M.; Frank, B.; Hermsen, M.; Deininger, M.H.; Dichgans, J.; Meyermann, R. Heat shock protein expression in human gliomas. *Anticancer Res.* **2000**, *20*, 4457–4462. [\[PubMed\]](#)
83. Borg, N.; Guilfoyle, M.R.; Greenberg, D.C.; Watts, C.; Thomson, S. Serum albumin and survival in glioblastoma multiforme. *J. Neurooncol.* **2011**, *105*, 77–81. [\[CrossRef\]](#) [\[PubMed\]](#)
84. Zhou, X.W.; Dong, H.; Yang, Y.; Luo, J.W.; Wang, X.; Liu, Y.H.; Mao, Q. Significance of the prognostic nutritional index in patients with glioblastoma: A retrospective study. *Clin. Neurol. Neurosurg.* **2016**, *151*, 86–91. [\[CrossRef\]](#) [\[PubMed\]](#)
85. Liu, J.; Dai, Y.; Zhou, F.; Long, Z.; Li, Y.; Liu, B.; Xie, D.; Tang, J.; Tan, J.; Yao, K.; et al. The prognostic role of preoperative serum albumin/globulin ratio in patients with bladder urothelial carcinoma undergoing radical cystectomy. *Urol. Oncol.* **2016**, *34*, 484.e1–484.e8. [\[CrossRef\]](#)

86. He, Z.Q.; Duan, H.; Ke, C.; Zhang, X.H.; Guo, C.C.; Al-Nahari, F.; Zhang, J.; Chen, Z.H.; Chen, Y.S.; Liu, Z.G.; et al. Evaluation of cumulative prognostic score based on pretreatment plasma fibrinogen and serum albumin levels in patients with newly diagnosed high-grade gliomas. *Oncotarget* **2017**, *8*, 49605–49614. [[CrossRef](#)] [[PubMed](#)]
87. Krenzlin, H.; Lorenz, V.; Alessandri, B. The involvement of thrombin in the pathogenesis of glioblastoma. *J. Neurosci Res.* **2017**, *95*, 2080–2085. [[CrossRef](#)]
88. Shankar, G.M.; Balaj, L.; Stott, S.L.; Nahed, B.; Carter, B.S. Liquid biopsy for brain tumors. *Expert Rev. Mol. Diagn.* **2017**, *17*, 943–947. [[CrossRef](#)]
89. Yekula, A.; Muralidharan, K.; Rosh, Z.S.; Youngkin, A.E.; Kang, K.M.; Balaj, L.; Carter, B.S. Liquid Biopsy Strategies to Distinguish Progression from Pseudoprogression and Radiation Necrosis in Glioblastomas. *Adv. Biosyst.* **2020**, e2000029. [[CrossRef](#)]
90. Saenz-Antonanzas, A.; Auzmendi-Iriarte, J.; Carrasco-Garcia, E.; Moreno-Cugnon, L.; Ruiz, I.; Villanua, J.; Egana, L.; Otaegui, D.; Sampron, N.; Matheu, A. Liquid Biopsy in Glioblastoma: Opportunities, Applications and Challenges. *Cancers* **2019**, *11*, 950. [[CrossRef](#)]
91. Piccioni, D.E.; Achrol, A.S.; Kiedrowski, L.A.; Banks, K.C.; Boucher, N.; Barkhoudarian, G.; Kelly, D.F.; Juarez, T.; Lanman, R.B.; Raymond, V.M.; et al. Analysis of cell-free circulating tumor DNA in 419 patients with glioblastoma and other primary brain tumors. *Cns. Oncol.* **2019**, *8*, CNS34. [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

DISCUSSÃO

A NEUROIMAGEM COMO BIOMARCADOR

O aparecimento do conceito de pseudoprogressão, inicialmente por Hoffman e col. em 1997 (Hoffman WF, 1997) e mais extensamente descrito em 2004 por Wit e col. (Wit MCY, 2004), definido como um fenómeno de alterações subagudas de imagem nos doentes com gliomas subsequentes ao tratamento de radioterapia e quimioterapia sugestivas de progressão, com ou sem sinais clínicos associados, que resolvem espontaneamente sem tratamento, levantou-nos a questão de quais os critérios mais adequados para a sua deteção e se a sua presença poderia ser um indicador de prognóstico nos doentes com glioblastoma. Caracteristicamente surge em 2 a 3 meses após o tratamento, podendo, contudo, ocorrer até aos seis meses ou progredir para necrose relacionada com o tratamento. Contudo, a sua incidência, o tempo de ocorrência e o seu impacto no prognóstico mantêm-se mal definidos.

De acordo com o primeiro objetivo fomos tentar esclarecer a frequência de pseudoprogressão após os tratamentos de quimiorradioterapia e avaliar a acuidade dos critérios imagiológicos de resposta. Avaliamos uma coorte de 117 doentes com glioblastoma tratados entre Janeiro de 2005 e Dezembro de 2009 de acordo com o Protocolo Stupp, dos quais 70 doentes estavam disponíveis para avaliação da resposta radiológica.

A avaliação do estado da doença era feita utilizando os critérios de MacDonald havendo, contudo, a sensibilidade que não responderiam cabalmente aos desafios que os novos tratamentos impunham. A introdução dos critérios de RANO (**Tabela 1**) veio tentar otimizar esta avaliação tirando alguma ambiguidade aos achados de imagem.

A avaliação da primeira ressonância magnética de controlo pós tratamento realizada após um mês da conclusão da fase concomitante do protocolo de Stupp mostrou que, quando eram usados os critérios de MacDonald, 42 doentes (60%) apresentavam uma progressão inicial e no seguimento subsequente, 32 doentes (76%) tiveram uma verdadeira progressão, enquanto 10 doentes (24%) foram considerados como tendo pseudoprogressão. Dos 28 doentes com doença não progressiva, 26 (93%) tiveram doença estável e 2 doentes (7%) resposta parcial.

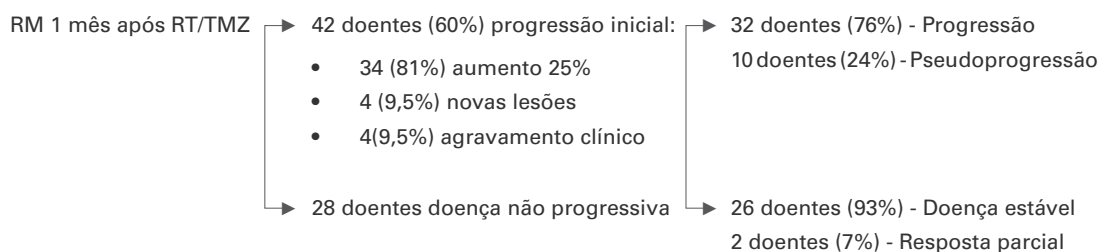
Tabela 1. Criteria for response assessment in high-grade gliomas.

Response	Criteria	
	MacDonald criteria	RANO criteria
Complete response	All: complete disappearance of all enhancing measurable and nonmeasurable diseases sustained for at least 4 weeks, no new lesions, no corticosteroids, and being stable or improved clinically.	All: T1 gadolinium enhancing disease, none; T2/FLAIR, stable or decreasing; new lesion, none; corticosteroids, none; clinical status: stable or improving.
Partial response	All: $\geq 50\%$ decrease in sum of products of perpendicular diameters of all measurable enhancing lesions sustained for at least 4 weeks, no new lesions, stable or reduced corticosteroid dose, and being stable or improved clinically.	All: T1 gadolinium enhancing disease, $\geq 50\%$ decrease; T2/FLAIR, stable or decreasing; new lesion, none; corticosteroids, none; clinical status: stable or improving.
Stable disease	All: being not qualified for complete response, or progression; being stable clinically.	All: T1 gadolinium enhancing disease $< 50\%$ decrease but $< 25\%$ increase; T2/FLAIR: stable or decreasing new lesion: none; corticosteroids: stable or decreasing; clinical status: stable or improving.
Progression	Any: $\geq 25\%$ increase in sum of the products of perpendicular diameters of enhancing lesions, any new lesion, or clinical deterioration.	Any: T1 gadolinium enhancing disease: $\geq 25\%$ increase; T2/FLAIR: increasing new lesion: none; corticosteroids: not applicable; clinical status: deteriorating.

RANO Response Assessment in Neuro-Oncology; **FLAIR** Fluid-attenuated inversion recovery.

A mesma avaliação, mas utilizando os critérios de RANO, mostrou progressão inicial em 15 doentes (21%), dos quais 13 doentes (87%) correspondeu a uma verdadeira progressão e apenas 2 doentes (13%) tiveram pseudoprogessão. Dos 55 doentes (79%) com doença não progressiva inicial, 27 doentes mantiveram doença estável, 1 doente (1,8%) resposta parcial e 27 doentes (49%) apresentaram aumento da captação de contraste na área de irradiação, tendo sido considerada doença estável (**Figura 1**).

Critérios de MacDonald



Critérios de RANO

15 doentes (21%) Progressão	→ 14 doentes - nova capt fora da área RT 1 doente - tumor na histologia	→ 13 doentes (87%) - Progressão 2 doentes (13%) - Pseudoprogessão
55 doentes (79%) Não progressiva	→ 27 doentes - doença estável 1 doente - resposta parcial 27 doentes - ↑ captação no campo da RT (doença estável)	

Figura 1. Resposta imagiológica de acordo com os critérios de MacDonald e os critérios de RANO.

Uma das conclusões deste estudo é que a incidência de pseudoprogessão depende do critério pelo qual é avaliada. Mostramos que a utilização de critérios melhor definidos para o fenómeno da pseudoprogessão diminuía o número de falsos positivos, ou seja, tiravam do grupo da pseudoprogessão doentes com progressão real, mas, mesmo assim, existia muita indefinição nalguns casos. Kucharczyk e col num estudo publicado em 2017, fizeram uma análise similar comparando a incidência de pseudoprogessão utilizando os três critérios de avaliação (MacDonald, RANO e RECIST). Encontraram uma incidência menor quando usados os critérios de RANO (15% dos doentes vs 19% com os critérios de MacDonald e RECIST) (Kucharczyk MJ, 2017). Aliado ao facto de na literatura estarem reportadas incidências de pseudoprogessão entre 6 e 65% dos doentes (Kucharczyk MJ, 2017; Park HH, 2015), percebemos que não há consenso sobre vários aspetos, desde o que representa até ao seu diagnóstico. É importante salientar que um diagnóstico incorreto de progressão tumoral pode levar à cessação precoce de um tratamento efetivo com repercussão negativa na sobrevivência. A confirmação patológica é pouco viável e a distinção clínica e imagiológica pode demorar bastantes semanas. Como o maior volume tumoral no glioblastoma pode ser prognóstico de pior sobrevivência global, iniciar o tratamento da recidiva numa fase precoce é essencial, pelo que a correta distinção entre pseudoprogessão e progressão é da maior importância. A introdução dos critérios de RANO em substituição dos de MacDonald foi um avanço, contudo, na minha opinião, não são suficientes para uma resposta cabal num número não desprezível de situações. Os critérios de resposta dos tumores sólidos (RECIST) não se adequam ao glioblastoma desde logo por não considerarem a RMT2/FLAIR, à semelhança dos critérios de MacDonald, mas não considerarem também o uso de corticosteroides, a evolução clínica e, principalmente, por não terem considerado o conceito de pseudoprogessão.

Há várias teorias que tentam explicar o fenómeno da pseudoprogessão. Os mecanismos propostos para a neurotoxicidade associada à radiação são a lesão vascular, a lesão glial e da substância branca e o impacto no sistema enzimático fibrinolítico (Zikou A, 2018). A irradiação do tecido cerebral e do tumor promove a facilitação da passagem de contraste através da barreira hematoencefálica, a

resposta inflamatória antitumoral, a produção de edema, a necrose de radiação ou a captação induzida pela irradiação (Kucharczyk MJ, 2017). Outra possível explicação é que a lesão induzida pela radiação no sistema nervoso central depende do aumento da permeabilidade capilar induzida pela radioterapia levando à transudação de fluido para o espaço intersticial e o consequente edema cerebral. Além disto, se a permeabilidade capilar for alterada, a lesão originada pela quimioterapia pode ocorrer mais cedo e ser mais grave, ou seja, a radioterapia pode aumentar a eficácia da quimioterapia maximizando a absorção do fármaco seja na membrana celular pela rotura da barreira hematoencefálica como através da alteração do metabolismo celular (Brandes A, 2008). Combinações de fatores como a apoptose das células endoteliais, a rotura da barreira hematoencefálica e a lesão rádica das células tumorais residuais, também têm sido descritos como fatores causais (Park HH, 2015). Foram também descritas uma pausa temporária na biossíntese de mielina devido a lesão de oligodendrócitos e reações inflamatórias locais como resultado do tratamento como fatores causais da pseudoprogressão (Xi J, 2021). Um outro conceito é o da libertação de citocinas que levam à angiogénese. A principal citocina secretada após a irradiação é o fator de necrose tumoral alfa (TNF- α) que regula positivamente outras citocinas que induzem a apoptose das células endoteliais, a ativação de astrócitos e o aumento da permeabilidade da barreira hematoencefálica. Também foi descrito o aumento da expressão do VEGF na substância branca após o tratamento de radioterapia, levando ao aumento da permeabilidade dos pequenos vasos e à formação de edema cerebral havendo uma relação entre a expressão do VEGF e a magnitude do edema (Zikou A, 2018). O facto de os anticorpos monoclonais anti-VEGF como o bevacizumab que levam a uma normalização da barreira hematoencefálica por diminuição da permeabilidade vascular com a consequente redução no grau de captação de contraste na ressonância magnética pelo tumor e uma diminuição no edema circundante suportam a intervenção do VEGF nos mecanismos de resposta à agressão terapêutica.

Alguns estudos consideraram que a pseudoprogressão era um critério de prognóstico, com os doentes com pseudoprogressão a apresentarem uma sobrevivência global aumentada. O nosso estudo não mostrou este benefício de sobrevivência entre os grupos de pseudoprogressão e doença não progressiva, apenas uma diminuição da sobrevivência global no grupo da progressão verdadeira. Estes resultados foram posteriormente corroborados por Kucharczyk e col. (Kucharczyk MJ, 2017) que demonstraram que a pseudoprogressão definida pelos critérios de MacDonald ou de RANO não trouxe uma melhoria prognóstica comparado com os doentes com doença estável. Com os resultados que obtivemos não podemos considerar a pseudoprogressão como um marcador de prognóstico nos doentes com glioblastoma.

Abre-se um desafiante caminho para a neuroimagem na procura de marcadores com importância prognóstica, nomeadamente em termos de estudos metabólicos quer por imagem de ressonância magnética quer por imagem de PET.

Outra questão que se levanta e que é aliciante do ponto de vista de investigação de translação é a relação do estado da metilação do gene promotor do *MGMT* com a incidência de pseudoprogressão. Brandes e col. encontraram na primeira RM efetuada 1 mês após o tratamento de RT + TMZ concomitante aumento da lesão em 50 doentes (48,5%) enquanto 53 doentes (51,5%) apresentaram doença não progressiva. Dos 50 doentes com aumento da lesão, 32 (64%) apresentaram pseudoprogressão e 18 (36%) progressão precoce. A metilação do gene promotor do *MGMT* foi encontrada em 21 (66%) dos doentes com pseudoprogressão e em 2 (11%) dos doentes com progressão precoce. Dos doentes com doença não progressiva, 13 (25%) apresentavam a metilação e 40 (75%) eram não metilados. Encontraram uma diferença significativa entre o grupo de doença não progressiva e o grupo de pseudoprogressão ($p=0.0002$), mas não entre o grupo de doença não progressiva e o grupo de progressão precoce ($p=0.23$) para o estado do promotor do gene *MGMT*. Assim, o estado do promotor do *MGMT* previu pseudoprogressão em 91,3% dos casos metilados (95% CI, 72% a 99%), mas previu progressão precoce em apenas 59% dos casos não metilados (95% CI, 38% to 76%). Em termos de sobrevivência livre de progressão, os doentes com pseudoprogressão tiveram uma SLP mais longa quando comparados com os doentes com doença não progressiva (20.7 vs 11.4 meses; $P=0.001$). Curiosamente, a extensão da remoção ($p=0,4$), a idade ($p=0.69$) e o estado funcional ($p=0.86$) não se correlacionaram significativamente com a sobrevivência livre de progressão nem foram fatores de prognóstico para a sobrevivência global. Encontraram uma sobrevivência mediana significativamente influenciada pela pseudoprogressão (38 meses vs 10,2 meses no grupo de doença progressiva vs 20,2 meses no grupo com doença não progressiva; $p<0.0001$) e pelo número de ciclos administrados de temozolamida (<6 ciclos vs ≥ 6 ciclos; 13.7 e 34.8 meses; $P<0.0001$). Na análise multivariada, a sobrevivência foi significativamente influenciada pelo estado de metilação do gene promotor do *MGMT* ($p<0.001$) e pela deteção de pseudoprogressão ($p=0.045$) (Brandes A, 2008). Park e col. num total de 101 doentes com glioblastoma com aumento da captação de contraste na ressonância magnética realizada após 4 semanas do final da fase concomitante do protocolo de Stupp encontraram pseudoprogressão em 45 doentes (44,6%) e doença não progressiva em 56 doentes (55,4%). A pseudoprogressão foi observada em 24 (61,5%) dos 39 doentes com a metilação do promotor do gene *MGMT* e em 21 (33,9%) dos doentes não metilados ($p<0.001$), corroborando os achados anteriores (Park HH, 2015).

Se a metilação do gene promotor do *MGMT* se mostrou um importante fator de prognóstico de pseudoprogressão, a extensão da remoção como influenciador da

pseudoprogressão mantém-se por esclarecer. E neste aspecto, o estudo anterior de Park e col. veio trazer alguma informação. Encontraram pseudoprogressão em 17 doentes (29,8%) com remoção completa, 19 doentes (55,9%) com remoção subtotal, 8 doentes (88,9%) com remoção parcial e em 1 doente (100%) com biópsia, tendo concluído que a extensão da remoção era um preditor independente de pseudoprogressão (Park HH, 2015). O modelo de risco proporcional de Cox mostrou que o estado da metilação do gene promotor do *MGMT* (HR 2.51, $p < 0.01$) e o grau de remoção tumoral (HR 2.99, $p < 0.01$) influenciaram significativamente a sobrevivência. Este resultado contrariou os achados de Brandes e col. que não encontraram a relação entre a extensão da remoção e a sobrevivência. Li e col, numa série de 234 doentes com glioblastoma, obtiveram uma incidência de pseudoprogressão de 26% (61 doentes) e, na análise multivariada, encontraram com fatores relacionados com a pseudoprogressão a remoção completa do tumor, metilação do gene promotor do *MGMT* e a localização não atingindo a zona subventricular. Encontraram adicionalmente um aumento da sobrevivência livre de progressão e da sobrevivência global nos doentes com pseudoprogressão relativamente aos doentes com doença não progressiva ou com verdadeira progressão (Li M, 2021). Uma explicação poderá relacionar-se com o fato de apresentarem um elevado número de doentes com a metilação do promotor do *MGMT* e o aumento da sobrevivência se dever a esta circunstância e não à pseudoprogressão. Na realidade acabaram por concluir que os doentes com progressão precoce associada a fatores conhecidos de melhor prognóstico, corresponderiam mais a pseudoprogressão, os que estavam associados a fatores de agravamento do prognóstico as alterações de imagem corroboravam a verdadeira progressão. Penso que deverá ser sempre dentro desta visão integrativa dos diferentes fatores que as conclusões devem ser tiradas e não apenas por um ou outro fator em particular. Li e col. atribuíram maior importância prognóstica à metilação do gene promotor do *MGMT* do que à localização frontal, mas acabaram por concluir que os tumores com localização frontal estavam mais associados à presença da metilação que os tumores noutras localizações. Se considerarmos, como demonstrado por Hegi, que os doentes com glioblastoma com a metilação do gene promotor do *MGMT* têm melhor sobrevivência global e associarmos aos achados de Brandes que os doentes com a metilação do promotor do gene *MGMT* apresentam uma maior taxa de pseudoprogressão, poderíamos especular que os doentes que apresentassem pseudoprogressão então teriam maior sobrevivência global, sendo a pseudoprogressão um marcador de prognóstico no glioblastoma. Na realidade há um conjunto de interligações que devem ser consideradas e que transformam o glioblastoma numa entidade única daquele doente, mais do que uma entidade geral dum grupo de doentes. A justificação que pequenos resíduos na parede da loja são os causadores da progressão mais precoce é muito redutora e não explica o papel fundamental da radioterapia pós-operatória, por exemplo, no tratamento destas lesões residuais, que foi o primeiro grande avanço de sobrevivência no tratamento

dos doentes com glioblastoma quando associada à remoção cirúrgica, além de não haver até ao momento, nenhum limite mínimo de tamanho de tumor residual abaixo do qual a presença de doença possa ser considerada sem importância.

Considerando-se que a reação das células tumorais à radiação seja um fator importante na gênese da pseudoprogressão e sendo o principal fator pelo qual o grau de remoção é um fator preditor da pseudoprogressão, então estaríamos a considerar que para além da zona de captação de contraste existiria uma queda abrupta do volume de células tumorais, o que não foi comprovado em estudos de biópsia das margens que mostram uma decaída progressiva deste volume, o que poderá estar na gênese de 80% das recidivas dos glioblastomas se darem na margem da loca cirúrgica e, por outro lado, o planeamento do tratamento de radioterapia ir para além das margens do tumor captante em ressonância. Mas mantem-se claro que a definição de pseudoprogressão e os critérios de diagnóstico para pseudoprogressão e progressão precisam de ser mais rigorosos e melhor estabelecidos, com recurso a uma conjugação de aspetos, deixando de ser apenas a alteração radiológica subsequente ao tratamento de quimiorradioterapia. Park e col. consideraram doença progressiva quando surgiam lesões fora ou dentro do campo de radiação que não desapareciam ou continuavam a aumentar e pseudoprogressão as lesões captantes de contraste que regrediam espontaneamente ou após um curso curto de corticosteroides. Consideraram apenas a utilização de sequências avançadas de ressonância magnética como a perfusão e a espectroscopia ou a realização de PET – FDG nas situações de diagnóstico equívoco entre progressão e pseudoprogressão. A questão é que, tirando as situações verdadeiramente inequívocas de aparecimento de novas lesões fora da área de irradiação da radioterapia, todas as outras situações de captação de contraste ou aumento da área de captação que ocorram entre 1 a 3 meses após o término da fase concomitante do protocolo de Stupp poderão corresponder a situações de pseudoprogressão independentemente dos critérios usados para definir o estado de doença – progressão ou doença não progressiva. É nossa convicção que vários parâmetros devem ser considerados na definição de pseudoprogressão e na escolha dos exames adequados à sua deteção. Em termos de imagem de ressonância magnética, em todos os momentos de avaliação, e de acordo com o estado da arte, o protocolo mínimo de avaliação deverá incluir as sequências ponderadas em T1 e T1 Gad, as sequências ponderadas em T2 e T2FLAIR, as sequências de difusão (DWI e mapa ADC) e perfusão (DCS-rCBV e rCBF) e a espectroscopia que deverá considerar a área captante e a área não captante mas com alteração de sinal em FLAIR. As recorrências tumorais parece apresentarem coeficientes de difusão aparente (ADC) significativamente menores que a radionecrose. O rCBV tem uma sensibilidade e especificidade de, respetivamente, 81,5% e 77,8% no diagnóstico de pseudoprogressão (Kong Ds, 2011). A avaliação sequencial deverá compreender o mesmo protocolo de imagem. Permanecendo

dúvidas a imagem de PET poderá ajudar, devendo ser ponderado o radiofármaco de acordo com o grau de remoção determinado na RM precoce pós-operatória, sendo que nas remoções completas poderá ser considerado o PET com 18F-FDG e nas remoções subtotais a utilização de aminoácidos (18-FDOPA ou metionina). Mesmo a conjugação destas duas técnicas de imagem não resolve a totalidade dos casos, sendo necessário o recurso a outros aspetos como a clínica e alguns exames laboratoriais que possam acrescentar informação útil. Como anteriormente dito, a incidência de pseudoprogressão é muito variável conforme os estudos, que se deve numa grande parte à variabilidade dos critérios de diagnóstico. A recomendação de muitos neuro-oncologistas e, inclusive, dos critérios de RANO é precaução na interpretação das imagens de ressonância magnética até ao 3.º ciclo de temozolamida adjuvante o que corresponde aos 3 meses após o fim da radioterapia. Esta situação veio complicar-se com a introdução da imunoterapia no tratamento dos tumores cerebrais, o que levou à introdução de novas linhas de orientação para avaliação imagiológica destes doentes, designadamente os critérios iRANO (*immunotherapy response assessment for neurooncology*). Com a aplicação de estudos radiômicos, King JY e col. colocaram a hipótese de que um vetor de recursos de alto rendimento obtido a partir de mapas ADC e CBV poderia melhorar o diagnóstico de pseudoprogressão relativamente aos dados convencionais de ressonância magnética, ao fornecer informações biológicas de agressividade e vascularização do tumor num período inicial precoce pós-tratamento e tentaram desenvolver e validar um modelo radiológico usando RM multiparamétrica para facilitar a diferenciação entre pseudoprogressão e progressão precoce em doentes com glioblastoma. Um total de 6472 características foram extraídas dos dados da ressonância magnética (1618 características de cada T1Gad, FLAIR, ADC e CBV) e identificaram 12 características randômicas significativas para diferenciar entre pseudoprogressão e progressão precoce. O modelo de randômico de RM multiparamétrica mostrou um aumento significativo no desempenho em relação aos modelos radiômicos simples baseados nas imagens convencionais ou de difusão. Este modelo radiômico de ressonância multiparamétrica também mostrou melhor desempenho do que o modelo baseado apenas na perfusão, mas a diferença não foi estatisticamente significativa. Com este estudo mostraram que o modelo radiômico de ressonância multiparamétrica apresentava um desempenho superior a qualquer modelo radiômico de imagem única ou qualquer parâmetro único de ADC ou CBV (King JY, 2019). Ao conseguirmos distinguir corretamente a pseudoprogressão de progressão precoce, poderemos passar a utilizar a resposta radiológica objetiva ou a sobrevivência livre de progressão como critério para avaliação da eficácia de um tratamento específico em vez de se continuar a considerar a sobrevivência global como o marco para esta avaliação. Na verdade, a sobrevivência global pode não medir diretamente o impacto de um tratamento pelos efeitos confundidores associados às múltiplas variáveis que atingem estes doentes.

OS MODELOS MOLECULARES COMO BIOMARCADORES

Os glioblastomas apresentam uma etiologia multifatorial sendo o resultado de interações entre a exposição ambiental e a susceptibilidade genética. O único fator ambiental comprovado como causa é a radiação ionizante. A susceptibilidade genética a estes tumores pode ter uma relação com alguns polimorfismos de genes envolvidos na reparação do DNA, na regulação do ciclo celular ou na resposta imunológica. Um fator importante na formação e desenvolvimento dos glioblastomas é a angiogénese. O *VEGF* é o principal fator angiogénico no desenvolvimento embrionário e no crescimento tumoral e é altamente regulado no glioblastoma, estando os seus recetores sobreexpressos nos vasos tumorais, além de estarem altamente expressos nas células tumorais em paliçada perinecróticas, mas com regulação negativa nas células tumorais adjacente aos vasos, sugerindo uma expressão dependente do oxigénio (Taslimi S, 2018). O *VEGF* é produzido por muitos tipos de células, incluindo células tumorais, macrófagos e plaquetas. As atividades do *VEGF* não se limitam ao sistema vascular e entram em funções fisiológicas normais, como formação óssea, a hematopoiese e a cicatrização de feridas. É um mitogénio específico das células endoteliais com capacidade de aumentar a permeabilidade vascular. O *VEGF* secretado pelas células tumorais hipóxicas é distribuído por todo o tumor por difusão e vai-se ligando aos recetores das células endoteliais da membrana plasmática, aumentando a sua expressão e levando a uma cascata de eventos que incluem a proliferação e migração de células endoteliais e o aumento da permeabilidade e fenestração dos vasos tumorais (Taslimi S, 2018). Surgiram alguns estudos que mostraram que os polimorfismos de nucleotídeo único (SNPs) do gene *VEGF* podiam contribuir para a modulação dos níveis séricos de *VEGF*, possivelmente influenciando o risco e o prognóstico de tumores sólidos, incluindo gliomas. Assim, foi nosso propósito estudar o papel dos polimorfismos do gene *VEGF* no risco e na sobrevivência de doentes com glioblastoma.

Estudos das variantes genéticas do fator de crescimento endotelial vascular

Para tentar dar resposta ao segundo objetivo, propusemo-nos clarificar o papel dos polimorfismos do *VEGF* na susceptibilidade e prognóstico do glioma.

A angiogenese é reconhecida como um evento chave na progressão natural dos gliomas. A neovascularização nos tumores cerebrais correlaciona-se diretamente

com a sua agressividade biológica, o grau de malignidade e recorrência clínica e inversamente com a sobrevivência pós-operatória (Fischer I, 2005). O *VEGF* e seus recetores (*VEGFR*) são componentes centrais no desenvolvimento e progressão do glioblastoma. O *VEGF* é o principal fator angiogénico no crescimento do tumor e está altamente sobre ativado no glioblastoma, estando os seus recetores sobreexpressos nos vasos tumorais (Plate KH, 2012), quando comparado com o cérebro normal (Samoto K, 1995). A expressão do gene *VEGF* parece estar intimamente correlacionada com a vascularização nos gliomas humanos levando a uma densidade vascular aumentada, bem como a um mau resultado clínico (Samoto K, 1995). Além da função angiogénica, aumenta a permeabilidade vascular (Sjöström S, 2011). Localiza-se no citoplasma tumoral nas células em pseudopalisada e no endotélio, predominantemente na área perinecrotica (Takano S, 2010). Além do efeito parácrino nas células endoteliais, o *VEGF* secretado pelas células dos astrocitomas tem um efeito autócrino no crescimento e viabilidade celular, situação mediada pelo *VEGFR2*, envolvendo a co-ativação das vias do c-Raf / MAPK, P13 K / Akt e PLC/PKC (Sjöström S, 2011; Knizetova P, 2008). De notar que o *VEGF* é um dos genes conhecidos para cuja perda de um único alelo resulta em letalidade embrionária devido à falha em formar uma vasculatura funcional (Takano S, 2010). A ativação transcripcional de *VEGF* é aumentada principalmente por meio da transcrição do fator HIF-1. Alterações genéticas como a ativação do EGFR e a mutação do *PTEN* levam a um aumento da expressão do *VEGF* promovendo a atividade angiogénica (Fischer I, 2005).

Os genes são constituídos por DNA que é composto por sequências complexas de nucleotídeos. As variações nessas sequências que ocorrem de forma estável na população, sendo encontradas com frequência igual ou superior a 1%, são denominadas polimorfismos genéticos. O polimorfismo de nucleotídeo único (SNP) é uma variação na sequência do DNA que afeta somente uma base (adenina, timina, guanina ou citosina) na sequência do genoma. O gene *VEGF* está localizado no cromossoma 6p21.3 e é constituído por oito exões. As regiões 5' e 3' não traduzidas (UTR) contêm elementos regulatórios importantes responsivos à hipóxia contribuem para uma elevada variabilidade na produção de *VEGF* nos tecidos (Di Stefano AL, 2014). Pelo menos 44 SNPs com relevância clínica já foram determinados (Djan I, 2019). Os polimorfismos deste gene foram associados a tumores do pulmão, colo-retais, mama, oral, ovário, gástrico e bexiga. Estudos de associação genómica (GWAS) mostraram que polimorfismos de nucleotídeo único (SNPs) estavam associados à suscetibilidade de desenvolvimento de gliomas. Vários SNPs do *VEGF* gene demonstraram afetar a expressão do gene (Jiang H, 2013).

Os estudos que mediram a concentração de *VEGF* no soro dos doentes com glioblastoma tiveram resultados divergentes. Salven e col. encontraram níveis séricos de *VEGF* elevados em doentes com tumores cerebrais de alto grau (Salven

P, 1997). Reynés e col. encontraram níveis séricos de *VEGF* duas vezes maiores em doentes com glioblastoma quando comparados com os controlos saudáveis (Reynés G, 2011). Stockhammer e col. não encontraram diferenças nas concentrações séricas entre os doentes e os controlos saudáveis (Stockhammer G, 2000). Salven e Reynés apenas avaliaram valores pré-operatórios, Stockhammer apresentou resultados apenas de 6 doentes. Sjöström e col. avaliaram o impacto das variações genómicas dos genes *VEGF* e *VEGFR* no prognóstico dos glioblastomas. No gene *VEGF* não encontraram SNPs associados à sobrevivência e no gene *VEGFR2*, encontraram 2 SNPs, um na região promotora (rs2071559) e um no intrão 1 (rs12502008), associado ao prognóstico do glioblastoma num total de 44 SNPs estudados (Sjöström S, 2011). Consideraram poder tratar-se de falsos positivos tendo em conta o número de falsos positivos esperados considerando que os testes foram realizados com um nível de confiança de 95%. Takano e col demonstraram um aumento da expressão do *VEGF* no tecido tumoral e no líquido quístico dos glioblastomas, mas não detetaram este aumento da expressão no soro dos doentes (Takano S, 2010). Jiang e col. numa série de 880 doentes com glioblastoma e 880 controlos mostraram que os doentes com glioblastoma tiveram uma significativa maior frequência dos genótipos -634CC e +936TT do que a população de controlo (Jiang H, 2013).

Para concretizar este estudo foi feita a colheita de sangue periférico e o DNA foi extraído dos leucócitos.

Em relação ao risco de desenvolvimento de glioma e glioblastoma, encontramos uma associação significativa entre o SNPs *VEGF* heterozigoto rs833061 e risco para glioma e glioblastoma, bem como os portadores da variante deste SNP tinham um aumento do risco de glioma e glioblastoma. Além disso, o heterozigoto rs3024994 foi associado ao aumento do risco de glioma e os portadores da variante rs25648 demonstram um risco aumentado para oligodendroglioma. Nenhuma associação estatisticamente significativa foi encontrada entre os SNPs do *VEGF* rs3025035, rs3025039, rs3025040, rs1005230, rs699947, rs833070, rs3025010, e variantes rs2010963 e risco de glioma, glioblastoma ou oligodendroglioma.

O SNP rs833061 está também associado ao risco de desenvolvimento de outros tumores. Wu e col. (Wu X, 2013) encontraram uma associação deste polimorfismo com o risco de desenvolvimento de carcinoma hepatocelular, mas não encontraram associação com o risco de desenvolvimento de hepatite B crónica. Da mesma forma que no glioblastoma, o *VEGF* desempenha um papel importante na fase inicial da carcinogénese hepática e a sua expressão aumenta gradualmente desde os nódulos displásicos de baixo grau para nódulos displásicos de alto grau para carcinoma hepatocelular (Wu X, 2013). A sobreexpressão do *VEGF*, comum em vários tipos de cancro, pode traduzir uma influência genética no seu desenvolvimento e

não apenas o resultado da sua produção pelas células neoplásicas em resposta ao ambiente hipóxico gerado, num contexto de sobre regulação.

O facto de não termos encontrado relação entre os SNPs rs699947 e o rs2010963 e o risco de glioma, como foi encontrado por Vasconcelos e col. (Vasconcelos VCA, 2019) pode dever-se ao número reduzido de casos da nossa amostra. Não podemos excluir o facto de podermos estar a falar de populações distintas e, por esse motivo, os resultados não serem concordantes. Por outro lado, tivemos resultados concordantes com o SNP rs833061 que mostrou um risco aumentado do desenvolvimento de desenvolvimento de glioma. Relativamente ao SNP rs1005230 em que não encontramos relação com o risco, o grupo de Vasconcelos encontrou um efeito protetor do risco de glioma. Este efeito protetor no nosso estudo foi conferido pelo heterozigoto rs3024994, à semelhança dos resultados de Li e col., sendo que este SNP também mostrou uma diminuição do risco de desenvolvimento de cancro da bexiga (Li R, 2011). O SNP rs2010963 tem mostrado resultados contraditórios, tendo sido associado a uma proteção no risco de crescimento de melanoma e de carcinoma hepatocelular, mas também a um risco aumentado de desenvolvimento de cancro do pulmão e da próstata (Li R, 2011). A associação do SNP rs3025039 com a diminuição dos níveis plasmáticos de VEGF com possível diminuição da formação de vasos sanguíneos encontrada por Li e col. não foi corroborada no nosso estudo.

A maioria dos estudos tem sido conduzida na população chinesa o que pode acarretar um viés populacional importante atendendo à variabilidade de frequência destes SNPs. A variabilidade dos alelos em cada SNP pode determinar maior ou menor produção de *VEGF*, com maior formação de vasos sanguíneos e aumento da susceptibilidade de desenvolvimento de glioblastomas. Também diferentes associações de polimorfismos poderão condicionar suscetibilidades diferentes pelo que deverão ser feitas análises combinadas para avaliação de efeitos sinérgicos.

O *VEGF* é um gene altamente polimórfico com mais de 20 SNPs potencialmente funcionais relatados em estudos de associação de risco de desenvolvimento de cancro. Poderemos pensar que genótipos dos vários SNPs em conjunto, podem ser mais informativos do que apenas um deles a prever a sua importância para o desenvolvimento de cancro.

Apesar dos estudos dos SNPs do *VEGF* em diferentes tipos de cancro, o seu papel fundamental no risco de glioblastoma ainda está por documentar, ficando como conclusão que as anomalias na formação dos vasos sanguíneos induzidas pelos diferentes polimorfismos do gene *VEGF* podem influenciar o risco e a agressividade dos glioblastomas. Doentes com níveis aumentados de *VEGF* no sangue foram associados a metastização à distância e a pior prognóstico (Djan I, 2019).

Quando usados os SNPs do *VEGF* para determinação do prognóstico dos doentes com glioblastoma encontramos que os SNPs rs3024994, rs2010963, e particularmente os portadores homozigotos de rs1005230 foram associados a um pior prognóstico para glioma e glioblastoma. Di Stefano e col. investigaram a associação do SNP rs2010963, localizado na região terminal não translacional do gene *VEGFA*, com a sobrevivência, a resposta ao bevacizumab (BVZ) e a toxicidade vascular. O SNP rs2010963 (G + 405C, G-634C) no 5'-UTR aumenta a expressão de *VEGFA* em ambos os níveis transcricional e translacional e pode hipoteticamente influenciar a agressividade do tumor ou a resposta a terapias anti-angiogénicas (Di Stefano AL, 2014). Estes resultados surgiram no nosso estudo, mostrando uma associação entre este SNP e uma diminuição da sobrevivência livre de progressão e da sobrevivência global. No entanto Djan e col. não obtiveram os mesmos resultados, não encontrando qualquer relação com a sobrevivência global numa fase inicial do seu estudo mas, numa fase subsequente, os resultados sugeriram que o polimorfismo + 405C> G *VEGF* (rs2010963) podia ser usado como marcador genético de prognóstico de sobrevivência global nos doentes com glioblastoma e ser um alvo potencial para terapia anti-VEGF (Djan I, 2019). O SNP rs2010963 que no nosso estudo se associou a uma diminuição da sobrevivência livre de progressão e sobrevivência global, foi associado a um atraso na progressão de melanoma e carcinoma hepatocelular, tumores também associados à sobreexpressão do VEGF. Os genótipos homozigotos rs699947, rs833061 e variante rs2010963 foram associados a um aumento na sobrevivência livre de progressão em doentes submetidos a tratamentos com bevacizumab, um anticorpo monoclonal que bloqueia a ação do VEGF. Curiosamente, estes genótipos conferiram, no nosso estudo, uma diminuição da sobrevivência livre de progressão e da sobrevivência global. No nosso estudo houve um número muito pequeno de doentes submetido a tratamento anti-angiogénico e daí os resultados, mas o que deve ser notado é a mudança prognóstica trazida com este tratamento. Os SNPs que conferem diminuição da sobrevivência poderão ter maior sensibilidade ao bloqueio farmacológico sendo alvos de eleição para tratamentos dirigidos, à semelhança com o que acontece nos tumores da mama com recetores HER2 positivos que respondem de forma muito marcada a fármacos como o trastuzumab, um anticorpo monoclonal humanizado, derivado do ADN recombinante que se liga com elevada afinidade ao recetor do fator de crescimento epidérmico humano tipo 2. Os SNPs rs3024994, rs1005230 e rs2010963 associaram-se de forma consistente a uma diminuição da sobrevivência livre de progressão e da sobrevivência global conferindo um pior prognóstico a estes doentes.

Neste sentido, alguns SNPs do *VEGF* poderiam ser considerados biomarcadores de risco e prognóstico no glioblastoma. Contudo, o *VEGF* tem um papel primordial noutras condições não neoplásicas que muitas vezes acompanham os doentes com

cancro como é o caso da diabetes. Devido à falta de consistência nos diferentes estudos, alguns com muito poucos casos, o *VEGF* não demonstrou ser um marcador de diagnóstico ou de sobrevivência. Talvez mais importante que um marcador de prognóstico, a sobreexpressão do *VEGF* e a determinação de alguns dos seus polimorfismos podem ser biomarcadores relevantes na decisão quanto à terapia anti-angiogénica.

Estudos das variantes de outros genes associados à proliferação vascular e ao crescimento tumoral

O gene *MGMT* (O6-metilguanina DNA metiltransferase) é um gene que codifica uma enzima reparadora do DNA conferindo resistência à quimioterapia com agentes alquilantes, como é o caso da temozolamida. A restauração deste DNA consome a proteína *MGMT* que a célula deverá repor. Níveis elevados da atividade do *MGMT* nas células tumorais criam um fenótipo resistente travando o efeito terapêutico dos agentes alquilantes sendo um determinante importante da falha do tratamento. O silenciamento epigenético do gene *MGMT* por metilação do promotor está associado à perda da expressão do *MGMT* e à consequente diminuição da atividade de reparação do DNA. Este silenciamento foi associado a maior sobrevivência global em doentes com glioblastoma que, além da radioterapia, receberam quimioterapia com agentes alquilantes como a temozolamida ou a carmustina (Hegi M, 2005). Um ensaio de fase III realizado por Stupp e col. mostrou que os doentes com GBM tratados com radioterapia combinada com temozolamida concomitante e adjuvante tinham um aumento da sobrevivência global quando comparados com os doentes tratados apenas com a radioterapia, estabelecendo um novo padrão no manejo desses tumores (Stupp R, 2005). Apesar de conhecidos alguns fatores de prognóstico como a idade, o estado funcional do doente e o grau de remoção tumoral, estes fatores não conseguem prever o prognóstico numa base individual. Hegi e col. mostraram que os doentes com glioblastoma que tinham o promotor do gene *MGMT* metilado apresentavam uma sobrevivência global mediana francamente aumentada quando comparados com os doentes sem a metilação, sendo esta metilação um fator independente de prognóstico favorável independentemente do tipo de tratamento (Hegi M, 2005). Havendo na literatura alguma controvérsia, questionamos a verdadeira implicação do *MGMT* como um marcador prognóstico de GBM e preditor específico de resposta aos esquemas de quimioterapia baseados na temozolamida. Neste contexto tentamos avaliar a frequência da metilação do gene promotor do *MGMT* numa coorte de doentes tratados uniformemente com temozolamida. O estado da metilação do promotor do *MGMT* foi determinado em 80 amostras tumorais, das quais 38 (47,5%) apresentavam a metilação, o que está de acordo com a literatura, que mostra presença de metilação em 40-50% dos casos

(Louis D, 2016). Uma frequência maior ocorre em glioblastomas com a mutação do gene *IDH1*, uma outra característica que confere melhor prognóstico. Contudo, esta mutação *IDH* está presente em apenas 20% dos glioblastomas. Há uma relação entre as mutações do *IDH* e a metilação do promotor do *MGMT* em que quase todos os doentes com mutação *IDH* apresentam metilação do *MGMT*, facto que parece dever-se à inibição competitiva do alfa-cetoglutarato dependente desoxigenase TET2 pelo oncometabolito-2'-hidroxiglutarato. Como resultado, ocorre uma hipermetilação global do genoma das células tumorais com uma mutação *IDH*, sendo que o promotor do gene *MGMT* está também sujeito a esta hipermetilação global (Christians A, 2019).

Usando o teste de *log-rank*, não foram detetadas associações estatisticamente significativas entre cada variável individual e a SLP ou SG dos doentes com glioblastoma ($P > 0,05$). Da mesma forma, o modelo proporcional multivariado de Cox não mostrou qualquer correlação estatisticamente significativa entre as variáveis estudadas e o prognóstico dos doentes ($P > 0,05$). No entanto, os doentes em que havia metilação do promotor do *MGMT* apresentaram um SG ligeiramente melhor (16 meses) em comparação com os doentes não metilados (13 meses) mas estas diferenças não mostraram significância estatística ($p = 0,583$ na análise univariada; $p = 0,617$ no modelo multivariado). O mesmo aconteceu para a sobrevivência livre de progressão e para a taxa de sobrevivência aos 2 anos.

Uma questão que poderá ser relevante é que, no nosso estudo, todos os doentes foram tratados de forma uniforme, independentemente das características genéticas e moleculares do tumor. Mas, a ter em conta a metilação do gene promotor do *MGMT* e condicionar a utilização de quimioterapia com agentes alquilantes aos doentes metilados, do ponto de vista clínico e ético não seria, na minha opinião, aceitável, uma vez que não está demonstrado que só estes doentes beneficiam com o tratamento. A utilização da temozolamida em doentes sem a metilação tem trazido algum benefício em termos de SLP e SG. O único grupo em que há evidência que deverá ser feito o ajuste terapêutico baseado na metilação é o dos doentes acima de 70 anos e que resulta mais da proteção contra os efeitos nocivos do tratamento do que do efeito mais benéfico dos tratamentos, uma vez que a sobrevivência é significativamente pior do que a observada em doentes mais jovens e o tratamento ideal em termos de eficácia e segurança permanece uma questão de debate. A EANO preconiza que neste grupo etário seja utilizada monoterapia em que os doentes com metilação do gene promotor do *MGMT* façam quimioterapia com temozolamida não devendo ser sujeitos a tratamento de radioterapia, ficando esta reservada para os doentes sem a metilação. Os subgrupos dos doentes sem metilação não mostraram benefício de sobrevivência com a adição da temozolamida à radioterapia, mas apresentavam toxicidade hematológica acrescida. No estudo

original de Hegi e col. embora a diferença na sobrevivência com base no estado de metilação do *MGMT* tivesse sido altamente significativa, mais da metade dos doentes *MGMT* metilados (com um resultado previsivelmente favorável) não sobreviveu por 2 anos, enquanto ~ 15% dos doentes com *MGMT* não metilado (com um resultado previsivelmente desfavorável) sobreviveram mais de 2 anos (Hegi M, 2005). Este ponto coloca uma tónica importante noutros fatores que devem ser tidos em conta, e que, hierarquicamente deverão ser estudados e relevados relativamente ao estado de metilação do gene promotor do *MGMT*. Tendo em linha de conta que vários polimorfismos de fatores de crescimento angiogénico como, os já atrás falados, polimorfismos do gene VEGF e polimorfismos de genes relacionados com o ciclo celular ou com fatores de supressão tumoral, que falaremos mais adiante, várias possibilidades se levantam relativamente ao prognóstico dos glioblastomas. Um outro aspecto é o facto de a metilação do gene promotor do *MGMT* ter sido associada com resultados melhores tanto nos doentes submetidos a quimiorradioterapia com base na temozolamida como nos doentes submetidos apenas a tratamento de radioterapia, sugerindo que o estado da metilação possa ser um marcador de prognóstico associado à natureza do tumor e não um preditor de resposta aos tratamentos. No nosso estudo, tentamos esclarecer se a metilação do gene promotor do *MGMT* era um biomarcador de resultado clínico nos doentes com glioblastoma tratados com o protocolo de Stupp e como ela se comparava com o valor prognóstico dos fatores clínico-patológicos clássicos. Não conseguimos detetar quaisquer correlações significativas entre as características clínicas dos doentes, o estado de metilação do *MGMT* e o prognóstico. Apesar doutros estudos terem resultados semelhantes ao nosso, estudos posteriores contrariaram estas conclusões. O reduzido número da amostra pode ser uma das razões dos resultados não terem atingido significância estatística. Poder-se-á dizer que o facto de ser multicêntrico tenha tornado a amostra menos homogénea, mas estamos a falar duma área demográfica pequena em que estas variações não poderão ser consideradas significativas. Contudo, num artigo de revisão, Rao e col. tentaram perceber o real significado prognóstico da metilação do gene promotor do *MGMT* e concluíram que, como biomarcador preditor, o estado da metilação pode ser afetado pela idade, género e raça. Na idade pediátrica (onde a metilação é mais rara) e nos idosos é um importante preditor da sobrevivência global. Relativamente à área geográfica, a metilação *MGMT* permaneceu um marcador significativo de SLP nas diferentes partes do mundo, mas não teve o mesmo significado relativamente à SG nos doentes asiáticos com a presença da metilação em comparação com os doentes nos EUA e na Europa. Da mesma forma, outra revisão sistemática mostrou que a metilação teve efeito significativo na SG na população asiática, mas não na SLP, enquanto teve um efeito significativo na SG e na SLP na população caucasiana (Rao AM, 2018). A biologia do glioblastoma é complexa e a metilação do *MGMT* não é o único biomarcador prognóstico. A conjugação de um conjunto de fatores

de prognóstico, nomeadamente fatores epidemiológicos como a idade, o gênero e a raça e fatores genéticos e moleculares como os diferentes polimorfismos e mutações de genes relacionados com a proliferação vascular, a replicação celular ou o sistema imunológico e a procura de novos marcadores de resposta terapêutica trarão respostas mais conclusivas.

A via do receptor do fator de crescimento epidérmico (EGFR) desempenha funções importantes na regulação do crescimento celular, apoptose e diferenciação, um processo que é rigidamente regulado em células epiteliais normais. A desregulação desta via, que pode ocorrer por mutações somáticas do *EGFR* (*EGFRvIII*) ou amplificações genéticas, por exemplo, foi implicada na génese de alguns tumores, entre os quais o glioblastoma. Além das alterações somáticas, também variantes polimórficas germinativas da via EGF/EGFR foram implicadas no risco do desenvolvimento tumoral, na resposta aos tratamentos e no prognóstico dos doentes. Aproximadamente 50% dos glioblastomas têm amplificação e sobreexpressão de *EGFR*, 40% dos quais expressam a forma mutante *EGFRvIII*, resultando na ativação constitutiva da via EGFR.

Estudos de associação genómica alargada identificaram cinco polimorfismos de genes associados à regulação do ciclo celular que se relacionaram com a susceptibilidade do desenvolvimento de gliomas.

Mantendo sempre a perspectiva de que os genes envolvidos na angiogenese e na replicação celular podiam ter um papel fundamental na susceptibilidade, risco e prognóstico de doentes com glioblastoma, o nosso grupo estudou diferentes polimorfismos dos genes envolvidos nestes mecanismos, *EGFR* (-216G/T, -191C/A, e (CA)_n), *RTEL1* (rs6010620), *CDKN2A/B* (rs4977756), *PHLDB1* (rs498872), *TERT* (rs2736100) e *CCDC26* (rs4295627).

Carpentier e col. (22) mostraram uma associação entre o alelo -216T do *EGFR* e o aumento do risco de glioblastoma. Da mesma forma, foram identificadas repetições polimórficas de sequências de microssatélites (CA)_n no primeiro intrão do *EGFR* e a actividade transcripcional do *EGFR* diminuía com o aumento do número de repetições sugerindo que este polimorfismo desempenhava também um papel na susceptibilidade ao glioblastoma. Tendo em conta o conhecimento generalizado da importância da via de sinalização do *EGFR* no glioblastoma, o nosso grupo avaliou a relevância dos polimorfismos *EGFR* -216G/T, -191C/A e repetições (CA)_n na susceptibilidade e prognóstico no glioblastoma. Não encontramos associação estatisticamente significativa entre as variantes do genótipo *EGFR* -216G/T e o risco de glioma. Em contraste, quando comparado com o genótipo de referência -191C/C, o genótipo heterozigoto -191C/A foi significativamente associado com o

aumento do risco de glioma. Mas já encontramos associações significativas entre o genótipo -191A/A e o risco de glioblastoma. Também o genótipo de repetição (CA)_n SS foi significativamente associado ao risco de glioblastoma. Como esperado e, à semelhança de outros estudos, os genótipos *EGF* +61A/G e G/G foram associados ao risco de glioma, mas em particular de oligodendroglioma, atingindo quase valor estatístico para glioblastoma. Analisando a relação destes polimorfismos com o prognóstico no subgrupo de doentes com glioblastoma, não foram encontradas associações entre as variantes do *EGFR* -216G/T e a sobrevivência dos doentes. Já o genótipo -191C/A foi significativamente associado a um aumento da sobrevivência global quando comparado com o homozigoto -191C/C. O conjunto dos resultados sugerem fortemente que os polimorfismos *EGFR* -191C/A e as repetições (CA)_n do intrão 1 corresponderiam a marcadores de prognóstico no glioblastoma enquanto as variantes -216G/T não se relacionavam com o prognóstico. As diferenças metodológicas entre os diversos estudos podem estar relacionadas com as discrepâncias nos resultados, mas o nosso estudo demonstrou que os genótipos de repetição homozigotos do intrão 1 (CA)_n conferiam maior susceptibilidade para o desenvolvimento de gliomas e o genótipo heterozigoto -191C/A era um marcador preditivo de pior sobrevivência nos doentes com glioblastoma. Choi e col. concluíram que a sobreexpressão de *EGFR* parecia correlacionar-se com o grau de glioma, sendo observada em cerca de 40-50% dos glioblastomas em comparação com 10-26% dos astrocitomas anaplásicos (Choi Y, 2013). A expressão do *EGFR* também se correlacionou com uma pior resposta à radioterapia (Barker FG, 2001). Estes estudos fizeram uma avaliação global do *EGFR*, não mostrando resultados de acordo com variantes polimórficas. Podemos concluir que um elevado nível sérico de *EGFR*, quando presente, poderá ser usado como preditor de prognóstico e resposta terapêutica nos doentes com glioblastoma.

Um outro importante fator na carcinogénese é o fator transformador de crescimento β (TGF- β). Esta citocina multifuncional está envolvida em vários processos imunomoduladores que desempenham funções fundamentais na proliferação, diferenciação e apoptose celulares, na angiogénese, na produção de matriz extracelular e na progressão tumoral. OTGF- β apresenta duas funções antagónicas, como mostrou Kaminska e col. (Kaminska B, 2013). Atua como um forte inibidor da proliferação de células epiteliais e astrócitos normais, mesmo em estádios pré-malignos do cancro, sendo um fator supressor tumoral, mas, nalguns tipos tumorais como nos gliomas de alto grau, na fase de transformação maligna, as células epiteliais tornam-se resistentes à sua ação, e passa a comporta-se como fator oncogénico contribuindo para o crescimento e invasão celular diminuindo a resposta imunológica contra o tumor. Transpondo os resultados obtidos da função dos polimorfismos conhecidos deste gene no cancro gástrico e da mama, o nosso grupo avaliou a sua relevância no risco de glioma e prognóstico dos doentes. Demonstrou-se que os polimorfismos do

gene *TGF-β*-509C/T (rs1800469) e 869T/C (rs1800470) não se associaram ao risco de desenvolvimento de gliomas. Já a análise de sobrevivência mostrou que o genótipo homozigótico -509TT se associou a sobrevivências globais mais elevadas quando comparado com os doentes portadores dos genótipos CC+CT. Também o genótipo homozigótico 869CC foi associado com aumento da sobrevivência global dos doentes com glioblastoma quando comparado com os genótipos 869TT+TC. Os resultados estão de acordo com o previamente descrito que o alelo -509T está associado a um aumento da atividade transcripcional quando comparado com o alelo -509C, levando a um maior aumento das concentrações séricas de TGF-β entre os homozigóticos TT do que nos heterozigóticos CT e, da mesma forma, o alelo 869C foi associado a concentrações séricas mais elevadas de TGF-β, com impacto na expressão da proteína TGF-β (Grainger D, 1999; Kallamani VG, 2005; Watanabe Y, 2002). Estes dados permitiram concluir que os polimorfismos do gene TGF-β podem ser marcadores de prognóstico relevantes, especificamente o TGF-β-509TT e 869CC, que estão associados a um aumento da sobrevivência. Uma questão curiosa é que foi mostrado por Canazza e col. que, *in vitro*, após irradiação, tanto o TGF-β quanto β1-integrina estão aumentados e a capacidade de invasão das células U87 é aumentada em ensaios de invasão de matrigel, sugerindo que o aumento do TGF-β pode estar associado a uma maior capacidade de invasão de células de glioblastoma após a irradiação (Canazza A, 2011), talvez por ativação do MMP-2 (Desmarais G, 2012) associado ao facto de poder exercer um efeito protetor contra a lesão induzida pela radioterapia (Birch JL, 2020). Outro ponto importante é que o TGF-β2 regula negativamente a expressão do antígeno HLA-DR em células de glioblastoma humano, facilitando seu escape imunológico aos linfócitos T. Esta atuação do TGF-β no sistema imune pode levar à adoção de estratégias imunoterapêuticas para suprimir a sinalização de TGF-β que poderão ser promissoras para melhorar o prognóstico dos doentes com glioblastoma. Embora vários estudos tenham demonstrado os potenciais benefícios em direcionar o tratamento para a via de sinalização do TGF-β no glioblastoma, os dados não foram consistentemente encorajadores. Podemos considerar um papel dual do TGF-β no prognóstico do glioblastoma, com a presença de alguns polimorfismos a conferir uma melhoria prognóstica, mas na maior parte das vezes o fator procarcinogénico sobrepõe-se nos diferentes domínios.

A função desempenhada por fatores imunossupressores e a secreção de moléculas imunossupressoras por gliomas de alto grau está bem documentada e há muito se supõe que estes fatores imunológicos desempenham um papel no risco de glioma (Zhou M, 2010). Os doentes com história conhecida de alergia apresentam um risco 1,5 a 4 vezes menor de desenvolverem gliomas (Wiemels JL, 2009; Linos E, 2007). Uma meta-análise realizada em 2007 mostrou uma redução acentuada no risco de glioma em pessoas que sofrem de atopia e doenças infecciosas, sugerindo que o aumento da vigilância imunológica poderá ser um fator protetor contra o

glioblastoma (Linos E, 2007). A IgE é a classe de anticorpos mais responsável pelo aparecimento de doenças alérgicas atópicas, tendo sido proposto um papel anti tumoral para IgE nos tumores sólidos (Fu SL, 2008; Lin Y, 2011). Os doentes com níveis séricos elevados de IgE apresentaram sobrevivências globais mais elevados do que os com níveis normais (Wrensch M, 2006).

Em termos de risco de desenvolvimento de glioma, os resultados foram mais discrepantes, com estudos a mostrarem um risco maior nos doentes com valores séricos normais de IgE quando comparados com indivíduos com valores elevados, havendo outros estudos a mostrarem um benefício de risco apenas quando o aumento é ligeiro ou borderline (25–100 kU/L), não se verificando nem com valores normais (<25 kU/L) nem com valores elevados (>100 kU/L) (Calboli FC, 2011). Apesar de alguma discrepância de resultados, a maior evidência é no sentido do fator protetor duma resposta imune mais efetiva ou previamente já ativada, como ocorre nas doenças atópicas e alérgicas.

O citomegalovírus humano (HCMV) não é considerado um vírus oncogénico, mas os produtos dos genes HCMV podem ser oncomoduladores e a sua expressão nas células tumorais pode acelerar a progressão influenciando a angiogénese, a replicação e a invasão celular e interferir com a imunomodulação. O nosso grupo considerou investigar o papel de alótipos GM da IGHG na etiopatogénese do glioma, considerando que esses alótipos modulam uma estratégia de imunoevasão do citomegalovírus humano. O receptor Fc γ codificado pelo HCMV (Fc γ R), usado pelo vírus para escapar das consequências efectoras da ligação do anticorpo anti-HCMV, tem afinidade diferencial para proteínas as IgG1 expressando alelos GM distintos, nomeadamente uma afinidade maior para as proteínas IgG1 que expressam os alótipos GM 3 +, 1-, 2- do que para aqueles que expressam os alótipos GM 17 +, 1 +, 2 +. O nosso grupo levantou a hipótese de que estes alelos GM podem representar fatores de risco ou proteção, respetivamente, para o desenvolvimento de glioma induzido pelo HCMV (Pandey JP, 2014). Os resultados mostraram que comparando os indivíduos que eram homozigotos para o alelo GM 17, os homozigotos GM 3 tinham duas vezes mais probabilidade e os heterozigotos GM 3/17 tinham três vezes mais probabilidade de desenvolverem gliomas. Resultados semelhantes foram alcançados quando combinados os dados correspondentes aos alelos GM 3 e GM 3/17 num modelo dominante. O genótipo GM 3/17 e a combinação de GM 3 e GM 3/17 foram associadas a um aumento de mais de 3 vezes o risco para glioma de alto grau. As análises de frequência de alelo também mostraram um risco aumentado para gliomas associado ao GM 3, o que levou a concluir que o alelo GM 3 pode apresentar risco para o desenvolvimento de glioma.

Uma das linhas de investigação em tumores cerebrais assenta na relevância dos polimorfismos genéticos no risco, prognóstico e resposta à terapêutica. Mesmo com resultados nem sempre consensuais ou conclusivos, tem-se conseguido associar um número crescente de polimorfismos. A maioria tem em comum associarem-se a três aspetos relevantes da gliomagenese: a proliferação vascular, a regulação do ciclo celular e a atividade imunológica/inflamatória. A tradução clínica destes achados tem sido parca e não se consegue, em termos individuais, prever o risco e o prognóstico para dado indivíduo, sendo fundamental procurar a outros níveis fatores acessíveis que funcionem como biomarcadores. Atendendo à pouca praticabilidade clínica, pese embora a determinação de alguns polimorfismos poder ser útil, a inconsistência dos resultados e a multiplicidade dos polimorfismos com potencial informativo torna esta linha de investigação pouco funcional do ponto de vista prático, não trazendo benefício real quer do ponto de vista clínico individual, quer mesmo do ponto de vista de grupo, não sendo exequível a sua determinação de per si para todos os doentes. Talvez a opção mais coriãl passe pela determinação de um grupo limitado de marcadores que em conjunto cumpram os requisitos para marcadores de prognóstico e possam servir de orientação terapêutica.

Han e col. sugeriram que, dado o mau prognóstico do glioblastoma, é essencial compreender os caminhos moleculares fundamentais que conduzem à formação do glioma, a fim de desenvolver novas estratégias terapêuticas para esta doença (Han J, 2020). Esta é uma discussão de longa data e, à medida que mais caminhos moleculares se vão descobrindo e mais estratégias terapêuticas se vão perfilando, como por exemplo, os estudos recentes com os inibidores do PD1/PD-L1, mais temos percebido que falta entender a ligação entre os mecanismos deste cancro em particular, a resposta terapêutica e a persistência das reduzidas taxas de sobrevivência. Os inibidores do PD-1 mostraram eficácia nalguns tipos de cancro. A avaliação da expressão do PD-L1 pode ser útil na previsão da resposta aos inibidores dos pontos de verificação imunológicos. O nivolumab e o pembrolizumab, dois inibidores do PD-1, mostraram eficácia terapêutica nalguns tumores sólidos, o que levou à sua aprovação nos Estados Unidos pela *Food and Drug Administration* (FDA). No entanto, nos estudos randomizados fase 3 *Checkmate 498* e *Checkmate 143*, o nivolumab não mostrou benefício na SG quando comparado com o tratamento padrão em doentes com glioblastoma inicial ou recorrente. Também o estudo fase 1b KEYNOTE-028 com o pembrolizumab mostrou resultados insatisfatórios nos doentes com glioblastoma recorrente com expressão do PD-L1 (Lombardi G, 2020).

Uma área que tem vindo a ser desenvolvida e com resultados noutros cancros é a das biópsias líquidas. É um método minimamente invasivo que poderá permitir identificar a recorrência precoce e ao avaliar a biologia molecular do tumor de forma longitudinal, prever o prognóstico e a resposta aos tratamentos. A biópsia líquida

passou a ser considerada um novo padrão de cuidados em doentes oncológicos, principalmente após a aprovação pela FDA em 2016 do primeiro teste sanguíneo para o estado da mutação do EGFR no cancro do pulmão de não pequenas células (Gatto L, 2021). Poder-se-á levantar a questão quanto à colheita de líquido cefalorraquidiano em vez de sangue periférico para a pesquisa de biomarcadores, uma vez que a sua circulação continua pode entrar em contacto com o tecido tumoral e transportar células e metabolitos tumorais. Na prática clínica tem-se observado que, mesmo em glioblastomas disseminados, a pesquisa de células malignas no liquor tem sido negativa na maior parte dos casos. A biópsia líquida é uma forma alternativa de examinar perfis moleculares de tumor podendo utilizar sangue, líquido cefalorraquidiano ou outros fluidos corporais para deteção e isolamento de células tumorais circulantes ou ADN tumoral circulante, que no caso do glioblastoma poderá ser uma opção de futuro. Contudo continuamos no âmbito do seguimento, não havendo a possibilidade de ser usado para diagnóstico numa fase precoce, uma vez que há a necessidade da determinação prévia do perfil molecular do tumor para ser possível encontrar células circulantes com o mesmo perfil.

Apesar de termos encontrado correlações de alguns polimorfismos com o prognóstico dos doentes com glioblastoma, pareceu-nos que o caminho de investigação futuro possa não ser este, essencialmente se tivermos em conta a praticabilidade clínica que é o nosso motivo primordial.

Em simultâneo com os estudos genéticos e moleculares, e trazendo uma tónica mais clínica, avançamos com estudos no âmbito do metabolismo e inflamação.

MODELOS METABÓLICOS E INFLAMATÓRIOS COMO BIOMARCADORES

Tendo por base o objetivo três, tentamos encontrar no sangue periférico alguns marcadores que pudessem ser associados ao prognóstico dos doentes com glioblastoma.

A literatura prévia demonstrou que o sistema imune tem um papel fundamental na supressão do desenvolvimento e crescimento dos tumores cerebrais primários. A inflamação crónica aumenta o risco de desenvolver alguns tipos de cancro e o microambiente impulsionado pelas células inflamatórias pode levar à iniciação e promoção do tumor (Reynés G, 2011). As células necróticas libertam sinais pró-inflamatórios para o microambiente tumoral que ativam as células imunológicas que acabam por levar à promoção tumoral promovendo angiogénese, proliferação celular e invasividade. A resposta inflamatória associada ao tumor tem o efeito paradoxal de aumentar a tumorigénese e a progressão, fornecendo moléculas bioativas a este microambiente tumoral, que incluem fatores de crescimento que sustentam a sinalização proliferativa, fatores de sobrevivência que limitam a morte celular tumoral e fatores pró-angiogénicos e enzimas modificadoras da matriz extracelular que facilitam ainda mais a angiogénese e a invasão. Perceber em que medida os biomarcadores hematológicos pré-operatórios refletem o microambiente tumoral podendo servir como marcadores de diagnóstico e prognóstico é extremamente difícil uma vez que os aspectos inflamatórios e imunológicos da biologia do glioblastoma e a resposta aos tratamentos são bastante complexos. No entanto há evidência de que um maior estado de inflamação se correlaciona com um pior prognóstico em muitas doenças neoplásicas. O recrutamento de leucócitos como parte de uma resposta inflamatória requer a passagem através das vênulas pós-capilares para os espaços de *Virchow-Robin*, seguida pela passagem através da glia para o neurópilo. Uma vez que a inflamação tenha sido estabelecida por meio do recrutamento e ativação de leucócitos, o privilégio imunológico do SNC é prejudicado por vários mecanismos, incluindo a quebra da barreira hematoencefálica e a estimulação imunológica mediada por citocinas locais (Ellingson BM, 2017). Os neutrófilos e linfócitos associados à inflamação participam na comunicação entre as células tumorais e o microambiente perilesional. Foi estabelecida uma correlação entre o grau do tumor e a infiltração de neutrófilos no tecido tumoral, bem como uma correlação entre a infiltração tumoral e a contagem de neutrófilos circulantes (Fossati G, 1999) e, embora o mecanismo não esteja ainda completamente percebido, parece estar estabelecido que os neutrófilos inibem o sistema imunológico e promovem o crescimento do tumor suprimindo a atividade

dos linfócitos e a resposta das células T, tendo sido levantada também a possibilidade de as células tumorais libertarem fatores quimiotáticos levando ao aumento da produção de neutrófilos no microambiente tumoral e no sangue periférico e, por sua vez, os neutrófilos circulantes contêm e produzem VEGF, TNF e outras citocinas que contribuem para a progressão tumoral. Por outro lado, os níveis aumentados de neutrófilos suprimem a atividade citolítica dos linfócitos levando a linfopenia ou à diminuição da função linfocítica (Weng W, 2018). Esta situação poderá explicar o facto de os doentes com linfopenia pré-tratamento apresentarem um pior prognóstico relativamente aos doentes com valores normais (Xi J, 2021). Ao serem relacionados os valores absolutos de linfócitos com os achados de ressonância magnética, foi verificado valores consistentemente mais baixos nos doentes com verdadeira progressão quando comparados com doentes com pseudoprogressão, contudo a análise de sobrevivência global revelou que para aqueles que tinham pseudoprogressão, a sobrevivência global não foi significativamente melhor do que para aqueles que tiveram verdadeira progressão. Este estudo acabou por mostrar que o número absoluto de linfócitos caiu significativamente após o tratamento do glioblastoma tanto no grupo da pseudoprogressão como no grupo dos doentes com verdadeira progressão do tumor e este efeito imunossupressor persistiu até à progressão documentada por ressonância magnética. Contudo, a contagem absoluta de linfócitos e as alterações desta contagem desde a linha de base até o pós-tratamento ou progressão documentada por ressonância magnética não demonstraram ter valor preditivo na diferenciação entre pseudoprogressão e verdadeira progressão do tumor. Além disso, os doentes que tiveram progressão tumoral verdadeira tiveram uma sobrevivência global não significativamente pior do que aqueles que tiveram pseudoprogressão. Daí que a contagem absoluta de linfócitos não pode ser considerada um biomarcador de progressão ou sobrevivência nos doentes com glioblastoma (Xi J, 2021). Também a linfopenia resultante do tratamento de quimiorradioterapia nos doentes com glioblastoma foi associada a uma diminuição da sobrevivência global (Yovino S, 2012). A inflamação sistêmica crónica e subsequente mielopoiese manifestando-se como neutrofilia é uma marca do cancro. A neutrofilia suprime a resposta imune antitumoral pela marginalização e apoptose dos linfócitos envolvidos na resposta imune adaptativa. A neutrofilia perpetua a tumorigenese, promovendo a metastização, a angiogénese e o extravasamento de células tumorais e endoteliais para a circulação (Garrett C, 2021).

O rácio neutrófilo-linfócito (RNL) é um fator hematológico marcador de inflamação sistêmica e vários estudos demonstraram uma associação entre um RNL mais alto e um pior prognóstico em muitas doenças malignas (Templeton AJ, 2014; Guthrie GJ, 2013), nomeadamente influenciando o prognóstico em doentes com cancro do ovário, colorretal, mama, pancreático, urotelial, de células renais e mieloma, embora o mecanismo subjacente a esta associação ainda não esteja bem esclarecido.

Weng mostrou que o RNL pré-operatório se correlacionava com o grau histológico dos gliomas, com o nível mais alto de neutrófilos e mais baixo de linfócitos a ser observado nos doentes com glioblastoma (Weng W, 2018). Esta correlação também foi encontrada no glioblastoma, sendo que na maioria dos estudos valores do $RNL \leq 4$ têm sido associados a melhor prognóstico, com aumento do tempo de sobrevivência global relativamente aos doentes com $RNL > 4$ que tem um pior prognóstico. No nosso estudo não encontramos o RNL como um fator independente de mau prognóstico quando avaliado todo a coorte, mas no subgrupo de doentes que completaram o protocolo Stupp, um RNL pré-operatório > 7 correlacionou-se com uma sobrevivência global menor. O valor do RNL pré-operatório também não mostrou influência na sobrevivência global no estudo de Garrett, o mesmo não acontecendo com o valor pós-operatório em que valores $> 5,07$ se associaram à diminuição da sobrevivência global (Garrett C, 2021). Yersal mostrou que os doentes com $NLR < 4$ tiveram uma sobrevivência global mediana melhor (10,7 vs. 7,8 meses) que os doentes com $NLR > 4$, mas esta diferença não foi estatisticamente significativa (Yersal O, 2018). Esses resultados não corroboraram os achados anteriores de Bambury e col. e de Han e col. que mostraram uma correlação significativa entre $NLR > 4$ e uma menor sobrevivência global (Han S, 2014; Bambury RM, 2013). Se nos reportarmos ao estudo de Zhao e col. verificamos que os doentes com GBM com $RNL > 1,706$ mostraram uma sobrevivência global significativamente mais baixa do que os doentes com um $RNL \leq 1,706$ (Zhao C, 2021). Isto levanta-nos outra questão que é a diferença de pontos de corte entre os vários estudos o que claramente se irá refletir na apreciação final dos resultados. Além do RNL avaliamos o rácio plaqueta-linfócito (RPL) e o número absoluto de neutrófilos, linfócitos e plaquetas antes da cirurgia, 24 horas após a cirurgia e imediatamente antes do início de tratamento de quimiorradioterapia. Medido como variáveis contínuas, não encontramos correlação entre o RNL, o RPL, o número absoluto de neutrófilos, linfócitos e plaquetas nos três tempos e a sobrevivência livre de progressão ou a sobrevivência global. Medido como variável categórica, também não encontramos uma correlação entre o RNL pré-operatório e a sobrevivência global, mas um $RNL \leq 5$ correlacionou-se com uma sobrevivência livre de progressão mais curta. Weng e col. encontraram com o aumento do grau do glioma uma contagem maior de neutrófilos e uma diminuição da contagem de linfócitos, bem como um aumento significativo do RNL. Numa análise multivariada a idade e o RNL mostraram uma associação independente com o grau do glioma. Verificaram também que o RNL podia ser um parâmetro discriminativo de glioblastoma, mas não de glioma de outro grau, sendo que um valor do RNL de 2,36 foi o ponto de corte para a previsão de glioblastoma, com uma sensibilidade de 70,5% e uma especificidade de 70,1% (Weng W, 2018).

A avaliação da diferença entre as contagens pós-operatório e contagens pré-operatórias dos RNL, RPL, neutrófilos, linfócitos e plaquetas, também não se correlacionou com

a sobrevivência livre de progressão ou a sobrevivência global. Uma situação inesperada que encontramos foi que o grupo de doentes submetidos a tratamento de quimiorradioterapia de acordo com o protocolo de Stupp tendeu a ter um RNL >5 enquanto que os doentes submetidos a outros regimes terapêuticos tenderam a apresentar um RNL ≤ 5 . No grupo de doentes que completou o protocolo de Stupp, a análise de regressão linear multivariada mostrou que a idade mais avançada ao diagnóstico, a multifocalidade do tumor e o envolvimento de áreas eloquentes se correlacionaram com uma sobrevivência global mais curta, enquanto que a multifocalidade do tumor se correlacionou também com uma diminuição da sobrevivência livre de progressão. Neste estudo não encontramos uma relação do RNL com a sobrevivência livre de progressão, mas um RNL >7 teve relação significativa com uma sobrevivência global mais curta. Vários fatores podem estar na origem dos resultados discrepantes encontrados. Não é fácil explicar o facto dos doentes com RNL ≤ 5 terem um menor tempo de sobrevivência livre de progressão, exceto por um viés estatístico devido ao reduzido número de doentes ou ao facto de as escolhas terapêuticas estarem condicionadas por fatores como a idade e o estado funcional, que acabam por estar, por sua vez, condicionadas à agressividade tumoral. Esta agressividade tumoral tem que ser interpretada em todas as suas vertentes. Ter em conta a associação com os aspetos imunológicos, como por exemplo o papel da IL-8 na gliomagenese e da sua relação positiva com o grau tumoral ou a supressão da resposta imunológica pelas células T reguladoras no microambiente tumoral. Também a IL-6 está implicada na gliomagenese, podendo ser um fator regulador do crescimento tumoral. É uma citocina importante na ativação de várias vias de sinalização pro-oncogénicas no glioblastoma e a sua amplificação associa-se a uma menor sobrevivência destes doentes quando comparados com doentes com glioblastoma sem esta amplificação. Também parece ser produzida pelas células do glioblastoma promovendo o crescimento autócrino. A sua expressão está associada a uma diminuição da sobrevivência global e a um aumento da neovascularização, infiltração inflamatória e edema peritumoral, havendo um processo dinâmico de conjugação destes fatores, angiogénese, aumento da permeabilidade vascular induzida por fatores tumorais, reação imunológica e processo inflamatório (Chang C-Y, 2005). A IL-6 induz a expressão de VEGF, a produção de imunoglobulinas, a ativação, crescimento e diferenciação das células T promovendo a inflamação tecidual. Está bem estabelecido que a via de sinalização da IL-6-STAT3, juntamente com outras citocinas inflamatórias, como IL1 β , IL-23 e TNF α , foi implicada na progressão do glioblastoma, contribuindo para a resistência do tumor e recorrência. O STAT3 faz a sobre regulação de genes que podem facilitar a sobrevivência do tumor, a angiogénese e a resistência à morte celular como o *VEGF* e o *TERT*. Está ativado numa elevada percentagem de glioblastomas e contribui para a tumorigenese ao inibir a apoptose (West AJ, 2028). Kohsaka mostrou que a inibição do STAT3 reduz os níveis de MGMT levando

à ressensibilização ao tratamento com a temozolamida (Kohsaka S, 2012). Shan encontrou um aumento da IL-6 no soro dos doentes com glioma quando comparado com os controlos saudáveis e os valores tinham uma relação direta com o grau do glioma. Viram também que os valores diminuía com a remoção cirúrgica, o que faz pensar numa resposta imunológica antitumoral (Shan Y, 2015). Ter em conta os aspetos genéticos e moleculares, como o estado da metilação do gene promotor do *MGMT*, o estado da mutação do gene *IDH* e aqui seria também importante fazer-se uma correlação com os diferentes polimorfismos que conferem melhor ou pior prognóstico. Ter em conta as características imagiológicas que poderão ser fatores de prognóstico, como por exemplo o volume tumoral, a percentagem de tumor necrótico e a percentagem de tumor não captante, a presença de picos muito aumentados de lactatos na espectroscopia e o *mismatch* T2-FLAIR (que traduz de forma indireta o estado da mutação *IDH*). Além disso, temos um número muito pequeno de doentes que não foram submetidos ao tratamento segundo o protocolo de Stupp. Fica a questão sobre o papel do tratamento concomitante de radioterapia com a temozolamida no processo inflamatório. Sabendo que o objetivo da temozolamida na fase concomitante é ser radiosensibilizante, ou seja, tornar o tecido tumoral e o leito cirúrgico mais respondedores à radioterapia, pode significar que retira parte da resposta protetora à agressão (neste caso terapêutica). Se associarmos a este raciocínio o facto dos doentes melhor respondedores terem uma taxa significativamente maior de pseudoprogressão e que este fenómeno representa uma resposta inflamatória reacional exacerbada que vai ajudar na resposta antitumoral, então ainda mais confuso se torna perceber o mecanismo de tumorigenese e de resposta terapêutica. Por um lado, o desenvolvimento de um estado inflamatório exacerbado contribuiria para a neovascularização e progressão tumoral, mas por outro ajudaria na resposta terapêutica. Wang e col. encontraram na sua coorte de doentes uma correlação entre um RNL $<3,2$ e melhor prognóstico e NLR $> 3,2$ e mau prognóstico (Wang Z, 2019). Auezova e col. mostraram que os gliomas *IDH1* *mutantes* estavam associados a valores inferiores do RNL (Auezova R, 2019) inferindo que esta mutação se associava a um nível mais baixo de inflamação crónica e, portanto, a um melhor prognóstico. Zadora e col. chegaram a resultados semelhantes, corroborando um maior RNL de acordo com o maior grau tumoral (Zadora P, 2015). No entanto e apesar destes resultados, numa letter ao editor alertamos para o facto de que relacionar os valores de RNL com os graus de malignidade da OMS pode ser uma limitação na maneira de usar este marcador. Está estabelecido que diferentes tipos de glioma têm diferentes prognósticos e o esforço da atualização de 2016 da classificação da OMS é exatamente tentar classificar os tumores em subtipos e graduá-los de acordo com o prognóstico. Seguindo esta linha de pensamento, achamos que a discussão deve ser centrada no efeito dos valores do RNL no prognóstico dos diferentes subtipos de glioma, especificamente naqueles que são mais comuns e agressivos como é o caso do

glioblastoma. O que pensamos é que se conseguirmos definir um subtipo concreto de glioblastoma, o RNL poderá funcionar como factor de prognóstico nesse subtipo particular e não numa forma transversal em todo o tipo de glioblastomas. A maioria dos glioblastomas *IDH* mutantes são secundários, surgindo dum glioma de menor grau prévio com características genéticas, moleculares, de imagem e clínicas favoráveis, tudo preditores duma resposta inflamatória reduzida. Curioso também no nosso estudo é o facto de que a relação do RNL com o prognóstico só foi significativa nos doentes que seguiram a terapia *standard*. Isso pode sugerir que parâmetros como RNL podem, no futuro, monitorizar as opções terapêuticas apontando para a necessidade de mudança de tratamento caso se mantenham parâmetros inflamatórios elevados.

Voltamos ao ponto da dualidade inflamatória perante o processo de tumorigenese. Usando o RPL como marcador inflamatório, não encontramos qualquer correlação com a sobrevivência livre de progressão ou com a sobrevivência global. Os nossos resultados foram concordantes com os de Yersal que também não mostraram diferença estatisticamente significativa na sobrevivência global entre os doentes com valores elevados e baixos do rácio plaqueta-linfócito (Yersal O, 2018). Já os resultados de Wang mostraram que o RPL tinha um valor prognóstico independente nos doentes com glioblastoma e que um valor elevado do RPL se associava a um pior prognóstico.

Continuando à procura de um biomarcador fomos olhar para outros marcadores inflamatórios no sangue periférico. A largura de distribuição dos glóbulos vermelhos (RDW) estima a variação do tamanho dos eritrócitos calculada como o coeficiente de variação, ou seja, reflete o grau de variação (anisocitose) do tamanho dos eritrócitos circulantes. Um RDW mais alto representa uma faixa de tamanho de glóbulos vermelhos (RBC) mais ampla, enquanto um valor menor indica homogeneidade de tamanho, sendo um indicador da homeostasia eritrocitária. A anisocitose está envolvida numa variedade de doenças como as doenças cardiovasculares, trombose, diabetes e cancro. Um elevado nível de RDW (anisocitose) é um marcador prognóstico negativo para estas doenças, sendo a inflamação o mecanismo principal. Um conjunto crescente de evidências sugere que um nível elevado de RDW também se correlacionou com o prognóstico para vários tipos de cancro, que incluíam os tumores do esófago, tumores gastrointestinais, tumores hepáticos, tumores pulmonares e tumores hematológicos. Este valor prognóstico do RDW parece ser independente da idade e do género do doente. Considerando que a análise RDW faz parte do estudo de sangue pré-operatório básico, torna-se uma variável de fácil acesso e universal e que o RDW pode ser um preditor de eventos adversos, independentemente do diagnóstico, fomos avaliar se poderia ser um fator prognóstico de sobrevivência nos doentes com glioblastoma.

Registamos o valor do RDW em dois momentos: imediatamente antes da cirurgia e na primeira consulta antes do início da quimiorradioterapia adjuvante ou radioterapia, 4 a 6 semanas após a cirurgia. O RDW pré-operatório médio foi de $13,0 \pm 0,72\%$, com valores variando de 11,5-15,4%. O valor médio do RDW pós-operatório foi de $14,1 \pm 1,33\%$, variando entre 12,3 a 20,1%. Quando comparados os dois valores, houve uma diferença estatisticamente significativa com um valor de p de 0,021. Usando uma análise sequencial dos pontos de corte de RDW pelas curvas de Kaplan-Meier, foi possível identificar que um RDW pré-operatório $> 14,5\%$ apresentou uma sobrevivência global menor (7, IC 95% 0,0-15,8 meses) quando comparado àqueles com RDW $\leq 14,5\%$ (16, IC 95% 14,8-17,2 meses, $p < 0,005$). Quanto ao RDW pós-cirurgia, os doentes com ponto de corte $> 15\%$ tiveram uma sobrevivência livre de progressão inferior (5, IC 95% 3,62-6,37) quando comparados com RDW $\leq 15\%$ (8, IC 95% 5,89-10,11, $p = 0,035$), não tendo havido qualquer relação do RDW pós-operatório com a sobrevivência global. A análise univariada mostrou uma associação entre RDW pré-cirurgia e a sobrevivência global. Esta relação com a sobrevivência global permaneceu na análise multivariada quando ajustada para as outras variáveis relacionadas. Kaisman-Elbaz e col. apresentaram, resultados semelhantes, em que os doentes com RDW $< 14\%$ apresentavam uma maior sobrevivência global mediana, em particular quando associado a valores normais de hemoglobina. O RDW pode também traduzir um marcador de anemia. Não há uma explicação cabal para a associação entre o RDW aumentado e um pior prognóstico, exceto pela associação do RDW com um estado pro-inflamatório. Uma revisão sistemática e meta-análise de 2017 que englobou 16 artigos e 4.267 doentes com cancro, embora nenhum destes artigos se referisse a doentes com glioblastoma, mostrou que o RDW elevado estava associado a uma baixa sobrevivência global (Hu L, 2017). Por outro lado, uma meta-análise realizada por Wang e col. em doentes com gliomas mostrou que os doentes com um RDW mais alto tinham taxas de sobrevivência global menores e que o RDW aumentado era um preditor independente de pior sobrevivência (Wang DP, 2020). O estudo de Bao e col. não mostrou esta associação, mas utilizou um ponto de corte de 12,5%, podendo esta diferença ser o responsável pela ausência de resultados, apesar de a diferença de sobrevivência global tivesse sido de 8 meses, o que acaba por representar um acréscimo de 1/3 ao tempo de sobrevivência global. Compreendemos que do ponto de vista estatístico esta diferença não seja significativa, mas do ponto de vista clínico individual é claramente vantajosa. Seria interessante avaliar estes resultados, usando o ponto de corte mais frequente nos estudos de 14%, até porque seria importante otimizar o valor de corte para ser usado como referência efetiva e não dependente do estudo em questão. Liang e col. encontraram na análise multivariada uma associação entre o RDW $< 14,10\%$ e uma melhor sobrevivência global quando comparados com os doentes com o RDW $> 14,10\%$ (Liang R, 2017), sendo os valores de 375 dias vs 293 dias, ou seja, estamos a falar duma sobrevivência global média de 12,5 meses para o melhor grupo. Este

resultado traduz um pior prognóstico em termos de sobrevivência global nos doentes com valores elevados e não um benefício prognóstico nos doentes com valores baixos. Quando comparados estes resultados com os do nosso estudo verificamos que a sobrevivência global no grupo do RDW >14,10% foi similar (9,7 meses vs 7 meses no nosso estudo) enquanto que no grupo do RDW < 14,10% a sobrevivência global dos nossos doentes foi significativamente maior (12,5 meses vs 16 meses no nosso estudo). Uma diferença que poderá ter relevância é que apenas 10% dos nossos doentes não fizeram tratamento adjuvante de radioterapia e/ou quimioterapia enquanto que no grupo de doentes de Liang 30% dos doentes não foram submetidos a tratamentos complementares. Outra questão relevante é que a maioria dos estudos avalia os valores pré-cirurgia do RDW e, no caso do nosso estudo, também cerca de 1 mês após a cirurgia, mas antes do início dos tratamentos de quimiorradioterapia. Estes estudos acabam por deixar de lado tudo o que se passa ao longo do percurso terapêutico, não havendo uma avaliação longitudinal nem comparação com outros potenciais marcadores inflamatórios e com os achados de ressonância magnética. Inferir que um valor basal pré-tratamento tem uma importância prognóstica per si não se entrando em linha de conta com o seguimento evolutivo, nomeadamente o tipo de tratamento adjuvante, parece ser demasiado redutor. Na realidade, não tem havido grande apetência no estudo de parâmetros eritrocitários no contexto do glioblastoma, sem que haja alguma explicação em particular, uma vez que há também uma correlação entre os eritrócitos e alguns parâmetros inflamatórios, bem como uma relação entre os valores da hemoglobina e o cancro. Tem sido levantada a hipótese que a diminuição da hemoglobina decorra da agressividade do tumor, mas continua por se demonstrar qual o real mecanismo, o que leva à questão se será causa ou consequência. Uma explicação poderia ser o facto de a anemia potenciar o ambiente hipóxico tumoral e peritumoral, promovendo a angiogenese via produção de HIF-1 e VEGF e o aumento da actividade das metaloproteinases da matrix extracelular promovendo o crescimento e invasão tumoral. O aumento do RDW deve-se também ao ambiente de hipóxia induzido pelas células tumorais que leva a um aumento da eritropoietina e à aceleração da produção de eritrócitos com libertação precoce de reticulócitos. Este ambiente de hipóxia contribui também para a manutenção dum estado inflamatório, com libertação de citocinas pelas células tumorais que levam a um stress oxidativo que contribui para um aumento da anisocitose por comprometimento da maturação dos eritrócitos. Este seria um processo similar ao que ocorre em outros cancros como por exemplo no hepatocarcinoma (Goyal H, 2017). Uma outra condição, para além dos estados inflamatórios, que cursa com valores aumentados do RDW é o estado de desnutrição, que acaba por levar a uma diminuição da produção de eritropoietina com prejuízo na maturação dos eritrócitos. Apesar do glioblastoma ser uma lesão confinada ao SNC poder-se-ia assumir que tivesse pouca repercussão sistémica, quer do ponto de vista inflamatório quer do ponto de

vista nutricional. Já vimos que há uma evidente tradução sistémica inflamatória pelas alterações traduzidas a nível do sangue periférico, podendo traduzir o efeito do glioblastoma a nível sistémico ou, pelo contrário, traduzir a resposta sistémica à presença do tumor cerebral. Esta é ainda uma questão que está em aberto.

Os lactatos são um produto metabólico final de tumores altamente proliferativos, como o glioblastoma, e resultam de um mecanismo de sobrevivência do tumor que, devido a uma elevada taxa de glicólise aeróbica, eflui o ácido láctico para o seu microambiente através de transportadores transmembrana de monocarboxilato, resultando na acidificação do meio com criação de áreas necróticas e facilitadoras da disseminação tumoral. A inibição desta função dificulta a invasão dos gliomas. A maior concentração de lactato encontra-se no centro das partes vitais do tumor. Ao contrário das células normais que sofrem glicólise ou fermentação quando o oxigénio é limitado, muitas células cancerosas utilizam níveis elevados de glicose a uma taxa rápida com produção concomitante de ácido láctico, mesmo na presença de oxigénio, um fenómeno conhecido como glicólise aeróbica ou efeito Warburg. Reparamos que um número significativo de doentes com tumores cerebrais submetidos à ressecção macroscópica cirúrgica ou biópsia apresentavam níveis altos de lactatos séricos (≥ 2 mmol / L) antes ou no início da cirurgia. Uma apreciação mais atenta levou a concluir que estes níveis elevados ocorriam com maior frequência nos glioblastomas do que nos gliomas de baixo grau ou nas metástases. Para avaliar a possibilidade de os lactatos séricos poderem ser um marcador de diagnóstico ou prognóstico dos doentes com tumores cerebrais, avaliamos 152 doentes submetidos a cirurgia de tumor cerebral num período de 10 anos, onde incluímos gliomas de baixo grau e gliomas de alto grau. A determinação dos lactatos séricos foi feita após colheita de sangue periférico após a indução anestésica e antes da incisão cirúrgica. Foram excluídos os doentes com disfunção renal ou hepática conhecida e com insuficiência cardíaca congestiva, bem como os doentes que tiveram períodos de instabilidade hemodinâmica com necessidade de medicação inotrópicas ou transfusão de sangue antes da colheita de sangue. Nos doentes com gliomas de baixo grau a concentração média de lactatos foi de 1,38 mmol/L (SD = 0,61) e em gliomas de alto grau de 2,03 (SD = 0,81). O nível médio de lactatos séricos pré-cirurgia foi de 1,79 mmol/L e 42,1% dos doentes tinham níveis elevados (≥ 2 mmol/L). Quando comparamos os doentes divididos de acordo com os valores séricos normais (< 2 mmol/L) e elevados (≥ 2 mmol/L) encontramos associações significativas entre o nível elevado e o maior grau tumoral e o género feminino. Encontramos também uma associação entre o uso de corticoides pré-operatórios e um menor valor de lactatos séricos. Quando fomos analisar a associação entre o grau do tumor e as concentrações séricas de lactatos pré-operatórios encontramos, numa análise de regressão logística univariada uma associação significativa entre o valor dos lactatos séricos pré-ressecção elevado

e um grau tumoral elevado, não tendo sido encontrada relação entre o grau do tumor e o volume ou o género do doente. Um maior grau tumoral foi associado a uma idade mais avançada. No modelo de regressão multivariada, tanto a idade avançada, o uso de corticosteroides e os níveis elevados de lactatos mantiveram a associação com o grau elevado do tumor, o que implica que, após o ajuste para todos os outros fatores, o aumento da concentração pré-operatória de lactatos é um preditor de gliomas de alto grau. Resultados idênticos foram reportados por Ioannoni e col. em que a hiperlactacidémia pré-operatória (> 2 mmol/l) foi mais frequente nos doentes com glioblastoma do que nos doentes com meningioma (65,34% vs 40,00%) (Ioannoni E, 2020). Adicionalmente, avaliaram também os valores séricos pós-operatórios, tendo encontrado também valores mais elevados nos doentes com glioblastoma quando comparados com os doentes com meningioma, além de terem encontrado uma relação estreita entre a lactacidémia pré-operatória e pós-operatória. Da mesma forma que no nosso estudo, não encontraram relação entre os valores dos lactatos com a idade do doente. Enquanto o nosso estudo mostrou que níveis aumentados estavam mais associados ao género feminino, este grupo não encontrou essa relação. Concordante com o nosso estudo foi a conclusão que o principal fator envolvido na hiperlactacidémia nos doentes submetidos a craniotomia é o fenótipo maligno do tumor. Mariappan e col. também encontraram uma correlação significativa entre o valor dos lactatos séricos basais e os tumores de alto grau, não tendo encontrado correlações entre os lactatos séricos basais e a idade, o género, o tamanho ou a localização do tumor. Estes resultados estão em linha com os nossos.

Ao se aprofundar a função dos lactatos, Thakur e col. mostraram que os lactatos produzidos pelas células do glioblastoma aumentavam a expressão do cluster de diferenciação 44 (células CD44) e a libertação de exossomas promovendo a progressão maligna do tumor (Thakur A, 2021). Uma hipótese é que o excesso de lactato intracelular deva ser secretado pela célula para manter a alcalinização intracelular e o ambiente extracelular ácido criado pelos lactatos serve para promover a invasão de células tumorais e a metastização e suprimir a vigilância imunológica, aumentando a sobrevivência das células tumorais e a resistência à apoptose.

Além dos valores aumentados de lactato que podem ser encontrados no sangue periférico, podemos também detetar na RM espectral os níveis elevados encontrados no glioblastoma, podendo este método funcionar também como marcador diagnóstico de progressão tumoral.

Perceber a cascata metabólica que contribui para a génese tumoral permite, não só travá-la como criar mecanismos ativos de correção. Por outro lado, aproveitar os

produtos resultantes destes processos metabólicos, identificá-los e quantificá-los, pode ser uma forma de diagnóstico, seguimento e avaliação de prognóstico.

A inflamação crónica, como processo associado a diversas condições, pode acompanhar-se por desequilíbrio hormonal, aumento da resistência à insulina e excesso de peso (Kolb R, 2016). Apesar de alguns estudos associarem o excesso de peso a um pior prognóstico nos doentes com cancro (Demark-Wahnefried W, 2012; Parekh N, 2012), esta associação no glioblastoma não está ainda bem definida, havendo evidência contraditória da relação do índice de massa corporal (IMC) com a sobrevivência dos doentes com glioblastoma. Num estudo prospetivo publicado em 2010, Jones e col. não encontraram associação entre o peso corporal e as taxas de sobrevivência em 1.259 doentes com glioblastoma (Jones LH, 2010). Siegel e col. reportaram uma diminuição da sobrevivência nos doentes com baixo peso e com obesidade na altura do diagnóstico de glioma de alto grau (Siegel EM, 2013). Potharaju e col. relataram aumento da sobrevivência nos doentes com glioblastoma e com aumento de peso e obesidade (Potharaju M, 2018). Assim, foi nossa intenção avaliar o impacto do IMC na sobrevivência livre de progressão e na sobrevivência global dos doentes com glioblastoma primário submetidos a tratamento cirúrgico para determinar se o IMC poderia constituir um fator de prognóstico independente. Foram avaliados 193 doentes, 116 homens (60,1%) e 77 mulheres (39,9%), com mediana de idade de 60 anos. 32 doentes eram obesos, 102 tinham excesso de peso e os restantes 59 doentes tinham peso normal. A sobrevivência global mediana para todos os doentes foi de 17,3 meses, enquanto a sobrevivência livre de progressão mediana foi de 7,5 meses. À data da última avaliação 25% dos doentes obesos, 12,7% dos doentes com excesso de peso e 3,4% dos doentes com peso normal estavam vivos, sendo que 6,3% dos doentes obesos e 5,9% dos doentes com excesso de peso não tinham evidência de doença da ressonância cerebral de controlo enquanto todos os doentes com peso normal tinham tido progressão da doença. Os nossos resultados vão de encontro aos de Potharaju e col. e o impacto prognóstico positivo do $IMC \geq 30 \text{ kg/m}^2$ teve uma significância estatística similar à idade jovem e à remoção completa do glioblastoma. O que parece suceder é que os doentes com IMC elevado têm maior probabilidade de desenvolverem cancro, mas após o seu desenvolvimento passa a ser um fator de melhor prognóstico. É o chamado paradoxo da obesidade. Numa tentativa de tentar perceber este paradoxo, Sanchez e col. num estudo de coorte de 5 estudos clínicos diferentes, tentaram objetivar as diferenças no transcriptoma entre o tecido tumoral e peritumoral nos doentes com carcinomas de células renais tanto no local primário como nas zonas de metastização em doentes com peso normal e obesos. As diferenças que encontraram forma no sentido do aumento da atividade angiogénica tumoral e da inflamação peritumoral nos doentes obesos (Sanchez A, 2019). Estes resultados surgem muito a contracorrente uma vez que que estes achados pro-inflamatórios deveriam contribuir para uma diminuição da

sobrevivência e não o seu contrário. Por outro lado, poderemos argumentar que o doente com maiores reservas de gordura terá uma capacidade maior de resistência à agressividade dos tratamentos de quimioterapia, sendo que o desgaste se inicia por aí e não pela estrutura proteica. Também parece existir uma relação inversa entre a expressão da sintetase de ácidos gordos (FAS) e o aumento da sobrevivência em doentes com cancro, sendo que este gene está sobexpresso nos doentes obesos. Pelo contrário, a sua sobrexpressão associa-se a uma maior agressividade tumoral e a um pior prognóstico uma vez que parece promover a proliferação celular e inibir a apoptose (Horiguchi A, 2008). A expressão da FAS é estimulada pelas vias de sinalização *EGF/EGFR*, que estão também bastante ativas no glioblastoma. O estado nutricional parece ter uma relação direta com o estado inflamatório. A inflamação leva à hipoalbuminémia pela diminuição da sua produção e aumenta a permeabilidade capilar com maior extravasamento da albumina para o espaço extracelular e consequente diminuição do efeito antioxidante contra antigénios tumorais e criação de condições para a proliferação celular aberrante. A inflamação causa também imunossupressão pela inibição dos linfócitos que são células efectoras essenciais na imunidade antitumoral, inibindo a proliferação, a invasão e a migração das células cancerígenas. O índice prognóstico nutricional (PNI), que conjuga o valor da albumina com o valor dos linfócitos, é outro marcador que pode ser usado para mostrar esta intrincada ligação entre o microambiente tumoral e a resposta imune sistémica, e foi comparado com o RNL enquanto biomarcador de prognóstico, tendo mostrado nalguns estudos, uma associação mais fiável com a sobrevivência global e com a sobrevivência livre de progressão, além de ser um valor preditivo positivo da eficácia do tratamento adjuvante, sendo que houve correlação significativa entre uma menor sobrevivência livre de progressão e um baixo PNI pós-operatório e entre uma pior sobrevivência global e um baixo PNI pré-operatório, mas estes resultados não se confirmaram na análise multivariada. Este trabalho esteve em consonância com outros resultados atuais que mostram que o PNI pré-operatório não é um preditor independente significativo de sobrevivência global nos doentes com GBM (Garrett C, 2021).

Apesar dos estudos exaustivos genéticos, moleculares e metabólicos, o seguimento dos doentes com glioblastoma continua a ser predominantemente clínico e imagiológico, baseado em imagens de ressonância magnética e PET, não havendo forma de diagnóstico precoce de evolução tumoral. Fazendo uma pesquisa exaustiva da literatura usando as bases de dados MEDLINE e Cochrane, entre janeiro de 2005 e dezembro de 2018, usando os termos "*glioma, serum biomarkers*", considerando apenas artigos em língua inglesa e dados coletados em glioblastomas humanos, identificamos 174 artigos. Destes, apenas 39 artigos abordavam especificamente potenciais biomarcadores séricos, 30 estudos eram prospetivos, 5 estudos retrospectivos e 4 estudos de revisão, correspondendo a 52 moléculas diferentes e 30 delas apenas eram referidas uma vez. Dividimos os biomarcadores em categorias

específicas, embora vários deles tenham várias funções e sendo estas categorias altamente inter-relacionadas:

- (a) proliferação vascular e crescimento celular;
- (b) sistema imune;
- (c) inflamação;
- (d) estado nutricional;
- (e) marcadores de coagulação.

Tabela 2. Serum biomarkers reported in the literature with potential application in glioblastoma.

Vascular proliferation	Cell growth	Inflammatory	Immune System	Coagulation	Nutritional
BMP-2 CXCL 10	α2HS glycoprotein BMP2	CD23 Albumin	CD14 CD23	Fibrinogen ETC	IGFBP-2 Albumin
Haptoglobin α2	Cathepsin D	Ratio N-L	IgE	Prothrombin factors	
Metallo-proteinase 9	CXCL10	Ratio P-L	Osteopontin	TF (tissue factor)	
Osteopontin	EGFR	Amyloid A1	Recoverin		
YKL-40	GFAP		TGF-β		
VEGF	Metallo-proteinase 9		TNF-α		
PDGF-b	Osteopontin		IL-6		
TGF-β	Recoverin				
TNF-α	PDGF-b				
TSPI	IGF-1 TGF-β TNF-α IL-8				

Dos vários potenciais biomarcadores de glioblastoma, o YKL-40 emergiu como particularmente promissor em vários estudos, sugerindo que pode ser útil como um preditor de prognóstico podendo vir a ter um papel no diagnóstico e monitorização de doentes com glioblastoma. Embora uma ativação significativa dos processos inflamatórios, de angiogénese, de proliferação celular e de coagulação tivessem sido demonstrados em doentes com glioblastoma, nenhum dos marcadores circulantes relatados pôde ser associado a um resultado clínico específico. Talvez por este motivo é que nenhum destes marcadores foi implementado na rotina clínica e, portanto, não tem valor clínico. A heterogeneidade da doença e do doente podem contribuir para a falta de consistência dos resultados, associado ao facto de muitos estudos terem um número reduzido de doentes e à falta de padronização na colheita de amostras,

diferentes métodos de quantificação e falta de validação de resultados. Também o facto de muitos dos biomarcadores terem sido colhidos apenas no pré-operatório, sem avaliação longitudinal ao longo do curso da doença, leva a que não possam ser considerados no seguimento evolutivo, não podendo ter valor de diagnóstico ou prognóstico. Por último, convém referir que um subconjunto importante de doentes com GBM tem níveis séricos normais destes marcadores. No contexto complexo de gliomagenese, progressão e recrescimento, com uma rede multifatorial envolvendo proliferação vascular, crescimento celular, fatores inflamatórios, imunológicos, de coagulação e nutricionais, tentar abordar apenas um fator que poderia funcionar como um biomarcador de diagnóstico, prognóstico ou estado da doença poderá ser impossível e apenas um conjunto de biomarcadores pode realmente fornecer dados adequados e informação mais precisa. No entanto, no momento, isto não é facilmente executável dada a duração do processo e os custos envolvidos, o que o torna inadequado para cada doente em particular.

No cumprimento do objetivo 4, servindo de base para novas investigações iniciamos:

- Um projeto de ciência básica com o objetivo de pesquisar no sangue periférico do DNA tumoral circulante. Este projeto está na fase de início tendo sido obtido colheitas de sangue periférico em 13 doentes e colheita de tecido tumoral em 7 doentes;
- Um projeto de investigação clínica que consiste num estudo prospetivo da avaliação sequencial dos valores hematológicos como fator de prognóstico nos doentes com glioblastoma;
- Um projeto de investigação clínica que consiste num estudo prospetivo da pseudoprogressão como fator de prognóstico nos doentes com glioblastoma.

E pretendemos fazer uma mudança de paradigma em termos de investigação de translação – em vez de estudar o que corre bem, estudar o que corre mal:

- Avaliar das características genéticas, moleculares e clínicas dos doentes com glioblastoma “*short survivors*” e compará-las com os “*normal*” e os “*long survivors*”.

CONSIDERAÇÕES FINAIS

O glioblastoma é um tumor complexo. É o tumor cerebral primário maligno mais frequente, mas apesar disso é um tumor raro na população. O seu comportamento biológico agressivo cria dificuldades na investigação clínica pelo seu rápido desenvolvimento com um período sintomatológico pré-tratamento muito curto, pela deterioração clínica e neurológica rápida, pelos períodos de seguimento curtos, pela ausência de marcadores específicos de evolução, pela fraca resposta aos tratamentos existentes e pela diferença de resultados entre os estudos laboratoriais e os ensaios clínicos.

A investigação básica tende a ter uma maior preponderância na procura de um diagnóstico mais específico, na melhor caracterização genética e molecular e na caracterização dos fatores de risco e de prognóstico. A investigação clínica tem-se dedicado mais à monitorização da evolução e à orientação terapêutica.

Torna-se imperioso a integração da investigação básica nos aspetos clínicos da mesma forma que a investigação clínica tem que passar a dar resposta aos aspetos básicos. Só assim poderemos ousar dar uma resposta mais eficaz a esta patologia. Apesar de todos os esforços desenvolvidos e dos 46340 resultados na *Pubmed* com o termo “glioblastoma”, a sobrevivência global destes doentes pouco melhorou nos últimos anos, o que tem mantido aberto o debate sobre sacrificar a maior sobrevivência em troca duma melhor qualidade de vida, uma vez que os tratamentos de segunda linha têm pouco a acrescentar em termos de sobrevivência global.

Muitos avanços foram dados ao nível genético e molecular, tendo sido determinados múltiplos fatores de risco de desenvolvimento e de prognóstico do glioblastoma, mas a sua aplicabilidade clínica tem sido muito baixa. Uma questão que permanece em aberto, mas que parece não merecer a atenção dos investigadores é o facto de os estudos efetuados com o bevacizumab terem mostrado um aumento da sobrevivência livre de progressão sem aumento da sobrevivência global. Isto levou a que a Agência Europeia do Medicamento não tivesse aprovado o uso do bevacizumab na recidiva, mas fica por explicar o motivo pelo qual estes doentes após a recidiva vivem menos que os doentes a quem não tinha sido administrado bevacizumab. O que explica que os doentes que têm uma primeira sobrevivência livre de progressão maior, apresentam uma segunda sobrevivência livre de progressão menor e vice-versa?

A necessidade de se encontrar um biomarcador de prognóstico e seguimento dos doentes com glioblastoma esteve na base do desenvolvimento desta dissertação. Testamos modelos imagiológicos, genéticos e metabólicos.

O critério fundamental no que diz respeito ao potencial valor clínico de um candidato a biomarcador de cancro é a consistência e força da associação entre o biomarcador e o resultado da doença de interesse, sendo necessário trazer uma informação adicional ou uma melhoria às ferramentas existentes.

Apesar de todos os esforços, até à data, não há biomarcadores no sangue ou no liquor para a deteção, acompanhamento ou prognóstico dos glioblastomas.

Mesmo marcadores com importância prognóstica, como a mutação do gene *IDH* ou a metilação do gene promotor do *MGMT*, não são capazes de prever a resposta a um tipo particular de tratamento.

Marcadores moleculares únicos são, na sua maioria, insuficientes para acompanhar a dinâmica de doenças como o cancro e deverão ser substituídos por perfis de marcadores. Quando em conjunto, as concentrações séricas de certas proteínas poderão indicar a presença de um glioblastoma com alta sensibilidade e especificidade.

A maioria dos marcadores estão envolvidos em vários processos fisiológicos e patológicos e não são específicos dos glioblastomas, acabando por contrariar o conceito de biomarcador.

O facto de a extensão da remoção permanecer um dos fatores de prognóstico mais importantes põe a tónica num aspecto puramente mecânico e independente das alterações genéticas e moleculares, bem como das características demográficas dos doentes como a idade, o género ou a raça. Mais importante é que o grau de remoção cirúrgico se mantém como fator de pronóstico importante na recidiva. Faz-nos levantar a questão se o parco acréscimo da sobrevivência global não poderá ter apenas relação com a melhoria da técnica cirúrgica e com a introdução de adjuvantes cirúrgicos como o microscópio cirúrgico, a neuronavegação, a ecografia intra-operatória ou a fluorescência com ácido 5-aminolevulínico. Na realidade o tratamento médico continua a levantar muitas dúvidas quanto à sua eficácia e poucas certezas quanto às melhores opções a utilizar. Questões como a duração do tratamento adjuvante com temozolamida, 6 ciclos ou 12 ciclos, a eficácia de fármacos anti-angiogénicos como o bevacizumab ou a utilização de imunoterapia estão ainda longe de serem resolvidas. Não falando da introdução dos tumor treating fields que trouxeram uma ligeira melhoria na sobrevivência global e que atuam, mais uma vez, numa forma física.

A persistência das incertezas é, mais que tudo, um fator motivador para a continuação da procura de respostas a cada vez mais dúvidas que se levantam e daí

ser absolutamente essencial a manutenção da investigação básica que pretendemos desenvolver a nível do ADN tumoral circulante, a mudança de paradigma na investigação de translação começando por perceber o que distingue os extremos avaliando as características genéticas, moleculares e clínicas dos “*short survivors*” e compará-las com os “*normal*” e os “*long survivors*” e a continuação da procura de biomarcadores clínicos em sangue periférico que permitam a monitorização da evolução da doença.

BIBLIOGRAFIA

- A** Amelot, A., P. De Cremoux, V. Quillien, M. Polivka, H. Adle-Biasette, J. Lehmann-Che, L. Françoise, A. F. Carpentier, B. George, E. Mandonnet and S. Froelich (2015). *IDH-Mutation Is a Weak Predictor of Long-Term Survival in Glioblastoma Patients*. PLoS One 10(7): e0130596.
- B** Bao, G., M. Wang, S. Guo, Y. Han and G. Xu (2010). *Association between epidermal growth factor +61 G/A polymorphism and glioma risk in a Chinese Han population*. J Int Med Res 38(5): 1645-1652.
- Bao, G., M. Wang, S. Guo, Y. Han and G. Xu (2011). *Vascular endothelial growth factor +936 C/T gene polymorphism and glioma risk in a Chinese Han population*. Genet Test Mol Biomarkers 15(1-2): 103-106.
- Barami, K., L. Lyon and C. Conell (2017). *Type 2 Diabetes Mellitus and Glioblastoma Multiforme-Assessing Risk and Survival: Results of a Large Retrospective Study and Systematic Review of the Literature*. World Neurosurg 106: 300-307.
- Berendsen, S., E. van Bodegraven, T. Seute, W. G. M. Spliet, M. Geurts, J. Hendrikse, L. Schoysman, W. B. Huiszoon, M. Varkila, S. Rouss, E. H. Bell, J. Kroonen, A. Chakravarti, V. Bours, T. J. Snijders and P. A. Robe (2019). *Adverse prognosis of glioblastoma contacting the subventricular zone: Biological correlates*. PLOS ONE 14(10): e0222717.
- Bethke, L., E. Webb, A. Murray, M. Schoemaker, C. Johansen, H. C. Christensen, K. Muir, P. McKinney, S. Hepworth, P. Dimitropoulou, A. Lophatananon, M. Feychting, S. Lönn, A. Ahlbom, B. Malmer, R. Henriksson, A. Auvinen, A. Kiuru, T. Salminen, A. Swerdlow and R. Houlston (2008). *Comprehensive analysis of the role of DNA repair gene polymorphisms on risk of glioma*. Hum Mol Genet 17(6): 800-805.
- Birch, J. L., B. J. Coull, L. C. Spender, C. Watt, A. Willison, N. Syed, A. J. Chalmers, M. K. Hossain-Ibrahim and G. J. Inman (2020). *Multifaceted transforming growth factor-beta (TGFβ) signalling in glioblastoma*. Cell Signal 72: 109638.
- Brandes, A. A., E. Franceschi, A. Tosoni, V. Blatt, A. Pession, G. Tallini, R. Bertorelle, S. Bartolini, F. Calbucci, A. Andreoli, G. Frezza, M. Leonardi, F. Spagnoli and M. Ermani (2008). *MGMT promoter methylation status can predict the incidence and outcome of pseudoprogression after concomitant radiochemotherapy in newly diagnosed glioblastoma patients*. J Clin Oncol 26(13): 2192-2197.
- Burghardt, I., E. Ventura, T. Weiss, J. J. Schroeder, K. Seystahl, C. Zielasek, D. Gramatzki and M. Weller (2021). *Endoglin and TGF-β signaling in glioblastoma*. Cell Tissue Res.
- C** Canazza, A., C. Calatozzolo, L. Fumagalli, A. Bergantin, F. Ghielmetti, L. Fariselli, D. Croci, A. Salmaggi and E. Ciusani (2011). *Increased migration of a human glioma cell line after in vitro CyberKnife irradiation*. Cancer Biol Ther 12(7): 629-633.
- Chang CY, MC Li, SL Liao, YL Huang, CC Shen and HC Pan (2005). *Prognostic and clinical implication of IL-6 expression in glioblastoma multiforme*. J Clin Neurosci 12(8): 930-933.
- Chen X, G Yang, D Zhang, W Zhang, H Zou, H Zhao, X Zhang and S Zhao (2014). *Association between the epidermal growth factor +61 G/A polymorphism and glioma risk: a meta-analysis*. PLoS One 9(4): e95139.
- Christians A, A Adel-Horowski, R Banan, U Lehmann, S Bartels, F Behling, A Barrantes-Freer, C Stadelmann, V Rohde, F Stockhammer and C Hartmann (2019). *The prognostic role of IDH mutations in homogeneously treated patients with anaplastic astrocytomas and glioblastomas*. Acta Neuropathol Commun 7(1): 156.
- Coomans MB, LDirven, NKAaronson, BG Baumert, M Van Den Bent, A Bottomley, AA Brandes, O. Chinot, C. Coens, T. Gorlia, U. Herrlinger, F. Keime-Guibert, A. Malmström, F. Martinelli, R. Stupp, A. Talacchi, M. Weller, W. Wick, J. C. Reijneveld and M. J. B. Taphoorn (2019). *Symptom clusters in newly diagnosed glioma patients: which symptom clusters are independently associated with functioning and global health status?* Neuro Oncol 21(11): 1447-1457.
- Costa, B. M., P. Ferreira, S. Costa, P. Canedo, P. Oliveira, A. Silva, F. Pardal, G. Suriano, J. C. Machado, J. M. Lopes and R. M. Reis (2007). *Association between functional EGF+61 polymorphism and glioma risk*. Clin Cancer Res 13(9): 2621-2626.
- Cote, D. J., M. K. Downer, T. R. Smith, S. A. Smith-Warner, K. M. Egan and M. J. Stampfer (2018). *Height, waist circumference, body mass index, and body somatotype across the life course and risk of glioma*. Cancer Causes Control 29(8): 707-719.
- Cote, D. J., B. A. Rosner, S. A. Smith-Warner, K. M. Egan and M. J. Stampfer (2019). *Statin use, hyperlipidemia, and risk of glioma*. Eur J Epidemiol 34(11): 997-1011.

Curtin, L., P. Whitmire, C. R. Rickertsen, G. L. Mazza, P. Canoll, S. K. Johnston, M. M. Mrugala, K. R. Swanson and L. S. Hu (2020). *Assessment of Prognostic Value of Cystic Features in Glioblastoma Relative to Sex and Treatment With Standard-of-Care*. *Front Oncol* 10: 580750.

D DiStefano, A. L., M. Labussiere, G. Lombardi, M. Eoli, D. Bianchessi, F. Pasqualetti, P. Farina, S. Cuzzubbo, J. Gallego-Perez-Larraya, B. Boisselier, F. Ducray, C. Cheneau, A. Moglia, G. Finocchiario, Y. Marie, A. Rahimian, K. Hoang-Xuan, J. Y. Delattre, K. Mokhtari and M. Sanson (2015). *VEGFA SNP rs2010963 is associated with vascular toxicity in recurrent glioblastomas and longer response to bevacizumab*. *J Neurooncol* 121(3): 499-504.

Disney-Hogg, L., A. Sud, P. J. Law, A. J. Cornish, B. Kinnersley, Q. T. Ostrom, K. Labreche, J. E. Eckel-Passow, G. N. Armstrong, E. B. Claus, D. Ilyasova, J. Schildkraut, J. S. Barnholtz-Sloan, S. H. Olson, J. L. Bernstein, R. K. Lai, A. J. Swerdlow, M. Simon, P. Hoffmann, M. M. Nöthen, K. H. Jöckel, S. Chanock, P. Rajaraman, C. Johansen, R. B. Jenkins, B. S. Melin, M. R. Wrensch, M. Sanson, M. L. Bondy and R. S. Houlston (2018). *Influence of obesity-related risk factors in the aetiology of glioma*. *Br J Cancer* 118(7): 1020-1027.

Djan, I., S. Lucic, M. Bjelan, N. Vuckovic, N. Vucinic, A. G. Morganti, M. Djan and M. Lucic (2019). *The VEGF gene polymorphism in glioblastoma may be a new prognostic marker of overall survival*. *J buon* 24(6): 2475-2482.

Dobran, M., D. Nasi, S. Chiriatti, M. Gladi, L. D. Somma, M. Iacoangeli and M. Scerrati (2018). *Prognostic Factors in Glioblastoma: Is There a Role for Epilepsy?* *Neurol Med Chir (Tokyo)* 58(3): 110-115.

E Ellingson, B. M., C. Chung, W. B. Pope, J. L. Boxerman and T. J. Kaufmann (2017). *Pseudoprogression, radionecrosis, inflammation or true tumor progression? challenges associated with glioblastoma response assessment in an evolving therapeutic landscape*. *J Neurooncol* 134(3): 495-504.

Ellingson, B. M., P. Y. Wen and T. F. Cloughesy (2017). *Modified Criteria for Radiographic Response Assessment in Glioblastoma Clinical Trials*. *Neurotherapeutics* 14(2): 307-320.

Esteller, M., J. Garcia-Foncillas, E. Andion, S. N. Goodman, O. F. Hidalgo, V. Vanaclocha, S. B. Baylin and J. G. Herman (2000). *Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents*. *N Engl J Med* 343(19): 1350-1354.

F Fischer, I., J. P. Gagner, M. Law, E. W. Newcomb and D. Zagzag (2005). *Angiogenesis in gliomas: biology and molecular pathophysiology*. *Brain Pathol* 15(4): 297-310.

G Garrett, C., T. M. Becker, D. Lynch, J. Po, W. Xuan, K. F. Scott and P. de Souza (2021). *Comparison of neutrophil to lymphocyte ratio and prognostic nutritional index with other clinical and molecular biomarkers for prediction of glioblastoma multiforme outcome*. *PLoS One* 16(6): e0252614.

Gatto, L., E. Franceschi, V. Di Nunno, A. Tosoni, R. Lodi and A. A. Brandes (2021). *Liquid biopsy in glioblastoma management: from current research to future perspectives*. *Oncologist*.

Gauthier, L. R., M. Saati, H. Bensalah-Pigeon, K. Ben M̄Barek, O. Gitton-Quent, R. Bertrand, D. Busso, M. A. Mouthon, A. Collura, M. P. Junier, H. Chneiweiss, J. R. Pineda and F. D. Boussin (2020). *The HIF1α/JMY pathway promotes glioblastoma stem-like cell invasiveness after irradiation*. *Sci Rep* 10(1): 18742.

Ghasimi, S., C. Wibom, A. M. Dahlin, T. Brännström, I. Golovleva, U. Andersson and B. Melin (2016). *Genetic risk variants in the CDKN2A/B, RTEL1 and EGFR genes are associated with somatic biomarkers in glioma*. *J Neurooncol* 127(3): 483-492.

Gu, J., Y. Liu, A. P. Kyritsis and M. L. Bondy (2009). *Molecular epidemiology of primary brain tumors*. *Neurotherapeutics* 6(3): 427-435.

H Hau, P., R. Stupp and M. E. Hegi (2007). *MGMT methylation status: the advent of stratified therapy in glioblastoma?* *Dis Markers* 23(1-2): 97-104.

Hegi, M. E., A. C. Diserens, T. Gorlia, M. F. Hamou, N. deTribolet, M. Weller, J. M. Kros, J. A. Hainfellner, W. Mason, L. Mariani, J. E. Bromberg, P. Hau, R. O. Mirimanoff, J. G. Cairncross, R. C. Janzer and R. Stupp (2005). *MGMT gene silencing and benefit from temozolomide in glioblastoma*. *N Engl J Med* 352(10): 997-1003.

Hegi, M. E., L. Liu, J. G. Herman, R. Stupp, W. Wick, M. Weller, M. P. Mehta and M. R. Gilbert (2008). *Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity*. *J Clin Oncol* 26(25): 4189-4199.

Horiguchi, A., T. Asano, T. Asano, K. Ito, M. Sumitomo and M. Hayakawa (2008). *Fatty acid synthase over expression is an indicator of tumor aggressiveness and poor prognosis in renal cell carcinoma*. *J Urol* 180(3): 1137-1140.

- Howell, A. E., J. W. Robinson, R. E. Wootton, A. McAleenan, S. Tsavachidis, Q. T. Ostrom, M. Bondy, G. Armstrong, C. Relton, P. Haycock, R. M. Martin, J. Zheng and K. M. Kurian (2020). *Testing for causality between systematically identified risk factors and glioma: a Mendelian randomization study*. BMC Cancer 20(1): 508.
- Howell, A. E., J. Zheng, P. C. Haycock, A. McAleenan, C. Relton, R. M. Martin and K. M. Kurian (2018). *Use of Mendelian Randomization for Identifying Risk Factors for Brain Tumors*. Front Genet 9: 525.
- Ioannoni, E., G. Grande, A. Olivi, M. Antonelli, A. Caricato and N. Montano (2020). *Factors affecting serum lactate in patients with intracranial tumors - A report of our series and review of the literature*. Surg Neurol Int 11: 39.
- Jiang, H., M. Lian, J. Xie, J. Li and M. Wang (2013). *Three single nucleotide polymorphisms of the vascular endothelial growth factor (VEGF) gene and glioma risk in a Chinese population*. J Int Med Res 41(5): 1484-1494.
- Jones, L. W., F. Ali-Osman, E. Lipp, J. E. Marcello, B. McCarthy, L. McCoy, T. Rice, M. Wrensch and D. Ilyasova (2010). *Association between body mass index and mortality in patients with glioblastoma multiforme*. Cancer Causes Control 21(12): 2195-2201.
- Kalakoti, P., R. D. Murray, J. Pettersson-Segerlind, H. Smeds and A. Nanda (2016). *Cochlear implants in the etiopathogenesis of glioblastoma - an interesting observation or independent finding?* Acta Neurochir (Wien) 158(5): 907-912.
- Karipidis, K., M. Elwood, G. Benke, M. Sanagou, L. Tjong and R. J. Croft (2018). *Mobile phone use and incidence of brain tumour histological types, grading or anatomical location: a population-based ecological study*. BMJ Open 8(12): e024489.
- Kawada, T. (2020). *Tea, coffee and risk of glioma*. Excli j 19: 1314-1315.
- Khaddour, K., T. M. Johanns and G. Ansstas (2020). *The Landscape of Novel Therapeutics and Challenges in Glioblastoma Multiforme: Contemporary State and Future Directions*. Pharmaceuticals (Basel) 13(11).
- Kim, J. Y., J. E. Park, Y. Jo, W. H. Shim, S. J. Nam, J. H. Kim, R. E. Yoo, S. H. Choi and H. S. Kim (2019). *Incorporating diffusion- and perfusion-weighted MRI into a radiomics model improves diagnostic performance for pseudoprogression in glioblastoma patients*. Neuro Oncol 21(3): 404-414.
- Knizetova, P., J. Ehrmann, A. Hlobilkova, I. Vancova, O. Kalita, Z. Kolar and J. Bartek (2008). *Autocrine regulation of glioblastoma cell cycle progression, viability and radioresistance through the VEGF-VEGFR2 (KDR) interplay*. Cell Cycle 7(16): 2553-2561.
- Kohsaka, S., L. Wang, K. Yachi, R. Mahabir, T. Narita, T. Itoh, M. Tanino, T. Kimura, H. Nishihara and S. Tanaka (2012). *STAT3 inhibition overcomes temozolomide resistance in glioblastoma by downregulating MGMT expression*. Mol Cancer Ther 11(6): 1289-1299.
- Kong, D. S., S. T. Kim, E. H. Kim, D. H. Lim, W. S. Kim, Y. L. Suh, J. I. Lee, K. Park, J. H. Kim and D. H. Nam (2011). *Diagnostic dilemma of pseudoprogression in the treatment of newly diagnosed glioblastomas: the role of assessing relative cerebral blood flow volume and oxygen-6-methylguanine-DNA methyltransferase promoter methylation status*. AJNR Am J Neuroradiol 32(2): 382-387.
- Kucharczyk, M. J., S. Parpia, A. Whitton and J. N. Greenspoon (2017). *Evaluation of pseudoprogression in patients with glioblastoma*. Neurooncol Pract 4(2): 120-134.
- Le Rhun, E., M. Preusser, P. Roth, D. A. Reardon, M. van den Bent, P. Wen, G. Reifenberger and M. Weller (2019). *Molecular targeted therapy of glioblastoma*. Cancer Treat Rev 80: 101896.
- Liang, R. F., M. Li, Y. Yang, Q. Mao and Y. H. Liu (2017). *Significance of Pretreatment Red Blood Cell Distribution Width in Patients with Newly Diagnosed Glioblastoma*. Med Sci Monit 23: 3217-3223.
- Linos, E., T. Raine, A. Alonso and D. Michaud (2007). *Atopy and risk of brain tumors: a meta-analysis*. J Natl Cancer Inst 99(20): 1544-1550.
- Lombardi, G., V. Barresi, S. Indraccolo, M. Simbolo, M. Fassan, S. Mandruzzato, M. Simonelli, M. Caccese, M. Pizzi, A. Fassina, M. Padovan, E. Masetto, M. P. Gardiman, M. G. Bonavina, M. Caffo, P. Persico, F. Chioffi, L. Denaro, A. P. Dei Tos, A. Scarpa and V. Zagonel (2020). *Pembrolizumab Activity in Recurrent High-Grade Gliomas with Partial or Complete Loss of Mismatch Repair Protein Expression: A Monocentric, Observational and Prospective Pilot Study*. Cancers (Basel) 12(8).
- Louis DN, O. H., Wiestler OD, Cavenee WK (2016). WHO Classification of Tumours of the Central Nervous System, International Agency for Research on Cancer (IARC) 69372 Lyon Cedex 08, France.

- Mariappan, R., L. Venkatraghavan, A. Vertanian, S. Agnihotri, S. Cynthia, S. Reyhani, T. Tung, O. H. Khan and G. Zadeh (2015). *Serum lactate as a potential biomarker of malignancy in primary adult brain tumours*. J Clin Neurosci 22(1): 144-148.
- Montemurro, N., P. Perrini and B. Rapone (2020). *Clinical Risk and Overall Survival in Patients with Diabetes Mellitus, Hyperglycemia and Glioblastoma Multiforme. A Review of the Current Literature*. Int J Environ Res Public Health 17(22).
- Namboodiri, A. M. and J. P. Pandey (2011). *Differential inhibition of trastuzumab- and cetuximab-induced cytotoxicity of cancer cells by immunoglobulin G1 expressing different GM allotypes*. Clin Exp Immunol 166(3): 361-365.
- Nelson, J. S., C. M. Burchfiel, D. Fekedulegn and M. E. Andrew (2012). *Potential risk factors for incident glioblastoma multiforme: the Honolulu Heart Program and Honolulu-Asia Aging Study*. J Neurooncol 109(2): 315-321.
- Omuro, A. and L. M. DeAngelis (2013). *Glioblastoma and other malignant gliomas: a clinical review*. Jama 310(17): 1842-1850.
- Pandey, J. P., N. Kaur, S. Costa, J. Amorim, R. Nabico, P. Linhares, R. Vaz, M. Viana-Pereira and R. M. Reis (2014). *Immunoglobulin genes implicated in glioma risk*. Oncoimmunology 3: e28609.
- Park, H. H., T. H. Roh, S. G. Kang, E. H. Kim, C. K. Hong, S. H. Kim, S. S. Ahn, S. K. Lee, H. J. Choi, J. Cho, S. H. Kim, K. S. Lee, C. O. Suh and J. H. Chang (2016). *Pseudoprogression in glioblastoma patients: the impact of extent of resection*. J Neurooncol 126(3): 559-566.
- Parsons, D. W., S. Jones, X. Zhang, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, I. M. Siu, G. L. Gallia, A. Olivi, R. McLendon, B. A. Rasheed, S. Keir, T. Nikolskaya, Y. Nikolsky, D. A. Busam, H. Tekleab, L. A. Diaz, Jr., J. Hartigan, D. R. Smith, R. L. Strausberg, S. K. Marie, S. M. Shinjo, H. Yan, G. J. Riggins, D. D. Bigner, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). *An integrated genomic analysis of human glioblastoma multiforme*. Science 321(5897): 1807-1812.
- Plate, K. H., A. Scholz and D. J. Dumont (2012). *Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited*. Acta Neuropathol 124(6): 763-775.
- Porter, A. B., D. H. Lachance and D. R. Johnson (2015). *Socioeconomic status and glioblastoma risk: a population-based analysis*. Cancer Causes Control 26(2): 179-185.
- Prabhakaran, N., D. C. Miller, N. S. Litofsky and S. R. Frazier (2019). *Extraneural Metastasis of Primary Glioma Occurring in a Setting of Occupational Ionizing Radiation Exposure*. Case Rep Neurol Med 2019: 1748739.
- Rao, A. M., A. Quddusi and M. S. Shamim (2018). *The significance of MGMT methylation in Glioblastoma Multiforme prognosis*. J Pak Med Assoc 68(7): 1137-1139.
- Rodriguez-Hernandez, I., S. Perdomo, A. Santos-Briz, J. L. Garcia, J. A. Gomez-Moreta, J. J. Cruz and R. Gonzalez-Sarmiento (2014). *Analysis of DNA repair gene polymorphisms in glioblastoma*. Gene 536(1): 79-83.
- Rogers, L. R., Q. T. Ostrom, J. Schroer, J. Vengoechea, L. Li, S. Gerson, C. J. Nock, M. Machtay, W. Selman, S. Lo, A. E. Sloan and J. S. Barnholtz-Sloan (2020). *Association of metabolic syndrome with glioblastoma: a retrospective cohort study and review*. Neurooncol Pract 7(5): 541-548.
- Samoto, K., K. Ikezaki, M. Ono, T. Shono, K. Kohno, M. Kuwano and M. Fukui (1995). *Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors*. Cancer Res 55(5): 1189-1193.
- Saunders, C. N., A. J. Cornish, B. Kinnnersley, P. J. Law, E. B. Claus, D. Ilyasova, J. Schildkraut, J. S. Barnholtz-Sloan, S. H. Olson, J. L. Bernstein, R. K. Lai, S. Chanock, P. Rajaraman, C. Johansen, R. B. Jenkins, B. S. Melin, M. R. Wrensch, M. Sanson, M. L. Bondy and R. S. Houlston (2020). *Lack of association between modifiable exposures and glioma risk: a Mendelian randomization analysis*. Neuro Oncol 22(2): 207-215.
- Saunders, C. N., A. J. Cornish, B. Kinnnersley, P. J. Law and R. S. Houlston (2021). *Searching for causal relationships of glioma: a phenome-wide Mendelian randomisation study*. Br J Cancer 124(2): 447-454.
- Schuss, P., F. Lehmann, N. Schäfer, C. Bode, E. Scharnböck, C. Schaub, M. Heimann, A. L. Potthoff, J. Weller, E. Güresir, C. Putensen, H. Vatter, U. Herrlinger and M. Schneider (2020). *Postoperative Prolonged Mechanical Ventilation in Patients With Newly Diagnosed Glioblastoma-An Unrecognized Prognostic Factor*. Front Oncol 10: 607557.

- Schwartzbaum, J., M. Edlinger, V. Zigmont, P. Stattin, G. A. Rempala, G. Nagel, N. Hammar, H. Ulmer, B. Föger, G. Walldius, J. Manjer, H. Malmström and M. Feychting (2017). *Associations between prediagnostic blood glucose levels, diabetes, and glioma*. *Sci Rep* 7(1): 1436.
- Seliger, C., C. Ricci, C. R. Meier, M. Bodmer, S. S. Jick, U. Bogdahn, P. Hau and M. F. Leitzmann (2016). *Diabetes, use of antidiabetic drugs, and the risk of glioma*. *Neuro Oncol* 18(3): 340-349.
- Shan, Y., X. He, W. Song, D. Han, J. Niu and J. Wang (2015). *Role of IL-6 in the invasiveness and prognosis of glioma*. *Int J Clin Exp Med* 8(6): 9114-9120.
- Shih, C. C., T. S. Lee, F. Y. Tsuang, P. L. Lin, Y. J. Cheng, H. L. Cheng and C. Y. Wu (2017). *Pretreatment serum lactate level as a prognostic biomarker in patients undergoing supratentorial primary brain tumor resection*. *Oncotarget* 8(38): 63715-63723.
- T** Takano, S., T. Yamashita and O. Ohneda (2010). *Molecular therapeutic targets for glioma angiogenesis*. *J Oncol* 2010: 351908.
- Tan, A. C., D. M. Ashley, G. Y. López, M. Malinzak, H. S. Friedman and M. Khasraw (2020). *Management of glioblastoma: State of the art and future directions*. *CA Cancer J Clin* 70(4): 299-312.
- Taslimi, S. and S. Das (2018). Chapter 26 - Angiogenesis and Angiogenesis Inhibitors in Brain Tumors. *Handbook of Brain Tumor Chemotherapy, Molecular Therapeutics, and Immunotherapy* (Second Edition). H. B. Newton, Academic Press: 361-371.
- Thakkar, J. P., T. A. Dolecek, C. Horbinski, Q. T. Ostrom, D. D. Lightner, J. S. Barnholtz-Sloan and J. L. Villano (2014). *Epidemiologic and molecular prognostic review of glioblastoma*. *Cancer Epidemiol Biomarkers Prev* 23(10): 1985-1996.
- Thakur, A., C. Xu, W. K. Li, G. Qiu, B. He, S. P. Ng, C. L. Wu and Y. Lee (2021). *In vivo liquid biopsy for glioblastoma malignancy by the AFM and LSPR based sensing of exosomal CD44 and CD133 in a mouse model*. *Biosens Bioelectron* 191: 113476.
- V** Valente Aguiar, P., B. Carvalho, R. Vaz and P. Linhares (2021). *Body mass index as an independent prognostic factor in glioblastoma*. *Cancer Causes Control* 32(4): 327-336.
- Vasconcelos, V. C. A., G. J. Lourenço, A. B. C. Brito, V. L. Vasconcelos, M. V. C. Maldaun, H. Tedeschi, S. K. N. Marie, S. M. O. Shinjo and C. S. P. Lima (2019). *Associations of VEGFA and KDR single-nucleotide polymorphisms and increased risk and aggressiveness of high-grade gliomas*. *Tumour Biol* 41(9): 1010428319872092.
- Viana-Pereira, M., D. A. Moreno, P. Linhares, J. Amorim, R. Nabiço, S. Costa, R. Vaz and R. M. Reis (2020). *Replication of GWAS identifies RTEL1, CDKN2A/B, and PHLDB1 SNPs as risk factors in Portuguese glioma patients*. *Mol Biol Rep* 47(2): 877-886.
- Vieira de Castro, J., C. S. Gonçalves, S. Costa, P. Linhares, R. Vaz, R. Nabiço, J. Amorim, M. Viana-Pereira, R. M. Reis and B. M. Costa (2015). *Impact of TGF- β 1 -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis*. *Tumour Biol* 36(8): 6525-6532.
- W** Wang, J. J., H. Wang, B. L. Zhu, X. Wang, Y. H. Qian, L. Xie, W. J. Wang, J. Zhu, X. Y. Chen, J. M. Wang and Z. L. Ding (2021). *Development of a prognostic model of glioma based on immune-related genes*. *Oncol Lett* 21(2): 116.
- Wang, P. F., H. W. Song, H. Q. Cai, L. W. Kong, K. Yao, T. Jiang, S. W. Li and C. X. Yan (2017). *Preoperative inflammation markers and IDH mutation status predict glioblastoma patient survival*. *Oncotarget* 8(30): 50117-50123.
- West, A. J., V. Tsui, S. S. Styli, H. P. T. Nguyen, A. P. Morokoff, A. H. Kaye and R. B. Luwor (2018). *The role of interleukin-6-STAT3 signalling in glioblastoma*. *Oncol Lett* 16(4): 4095-4104.
- Wiemels, J. L., D. Wilson, C. Patil, J. Patoka, L. McCoy, T. Rice, J. Schwartzbaum, A. Heimberger, J. H. Sampson, S. Chang, M. Prados, J. K. Wiencke and M. Wrensch (2009). *IgE, allergy, and risk of glioma: update from the San Francisco Bay Area Adult Glioma Study in the temozolomide era*. *Int J Cancer* 125(3): 680-687.
- X** Xavier-Magalhães, A., A. I. Oliveira, J. V. de Castro, M. Pojo, C. S. Gonçalves, T. Lourenço, M. Viana-Pereira, S. Costa, P. Linhares, R. Vaz, R. Nabiço, J. Amorim, A. A. Pinto, R. M. Reis and B. M. Costa (2017). *Effects of the functional HOTAIR rs920778 and rs12826786 genetic variants in glioma susceptibility and patient prognosis*. *J Neurooncol* 132(1): 27-34.

Xi, J., B. Hassan, R. G. N. Katumba, K. Khaddour, A. Govindan, J. Luo, J. Huang and J. L. Campian (2021). *The predictive value of absolute lymphocyte counts on tumor progression and pseudoprogression in patients with glioblastoma*. BMC Cancer 21(1): 285.

Y Yang, B., L. Heng, S. Du, H. Yang, T. Jin, H. Lang and S. Li (2015). *Association between RTEL1, PHLDB1, and TREH Polymorphisms and Glioblastoma Risk: A Case-Control Study*. Med Sci Monit 21: 1983-1988.

Yersal, Ö., E. Odabaşı, Ö. Özdemir and Y. Kemal (2018). *Prognostic significance of pre-treatment neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio in patients with glioblastoma*. Mol Clin Oncol 9(4): 453-458.

Yovino, S. and S. A. Grossman (2012). *Severity, etiology and possible consequences of treatment-related lymphopenia in patients with newly diagnosed high-grade gliomas*. CNS Oncol 1(2): 149-154.

Z Zadora, P., W. Dabrowski, K. Czarko, A. Smolen, E. Kotlinska-Hasiec, K. Wiorkowski, A. Sikora, B. Jarosz, K. Kura, R. Rola and T. Trojanowski (2015). *Preoperative neutrophil-lymphocyte count ratio helps predict the grade of glial tumor - a pilot study*. Neurol Neurochir Pol 49(1): 41-44.

Zhang, S., Z. Ye, X. Song, G. Chen, C. Huai, Q. Wang, J. Song, D. Lu, Y. Zhao and H. Chen (2015). *Association of EFEMP1 gene polymorphisms with the risk of glioma: A hospital-based case-control study in a Chinese Han population*. J Neurol Sci 349(1-2): 54-59.

Zhao, C., L. Li, X. Guo, D. Song, M. Wang, Y. Zhai, F. Yang, Y. Xue and X. Wei (2021). *Preoperative Predictors of Early Mortality Risk in Isocitrate Dehydrogenase-Wild-Type Glioblastoma Patients Treated with Standard Therapy*. Cancer Manag Res 13: 1159-1168.

Zhou, P., L. Wei, X. Xia, N. Shao, X. Qian and Y. Yang (2014). *Association between telomerase reverse transcriptase rs2736100 polymorphism and risk of glioma*. J Surg Res 191(1): 156-160.

Zikou, A., C. Sioka, G. A. Alexiou, A. Fotopoulos, S. Voulgaris and M. I. Argyropoulou (2018). *Radiation Necrosis, Pseudoprogression, Pseudoresponse, and Tumor Recurrence: Imaging Challenges for the Evaluation of Treated Gliomas*. Contrast Media Mol Imaging 2018: 6828396.

ANEXOS

1 PROGNOSTIC VALUE OF *MGMT* PROMOTER METHYLATION IN GLIOBLASTOMA PATIENTS TREATED WITH TEMOZOLOMIDE-BASED CHEMORADIATION: A PORTUGUESE MULTICENTRE STUDY

Bruno M Costa,¹ Cláudia Caeiro,² Inês Guimarães,¹ Olga Martinho,¹ Teresa Jaraquemada,² Isabel Augusto,² Lúcia Castro,³ Lúcia Osório,⁴ Paulo Linhares,⁵ Mrinalini Honavar,⁶ Mário Resende,⁷ Fátima Braga,⁸ Ana Silva,⁹ Fernando Pardal,⁹ Júlia Amorim,¹⁰ Rui Nabeço,¹⁰ Rui Almeida,¹¹ Carlos Alegria,¹¹ Manuel Pires,¹² Célia Pinheiro,¹³ Ernesto Carvalho,¹³ José M Lopes,^{3,14,15} Paulo Costa,¹⁶ Margarida Damasceno,² Rui M Reis¹

¹Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, Braga; Departments of ²Oncology, ³Pathology, ⁴Radiology, ⁵Neurosurgery, Hospital S. João, Porto; Departments of ⁶Pathology, ⁷Neurosurgery, ⁸Oncology, Hospital Pedro Hispano, Matosinhos; Departments of ⁹Pathology, ¹⁰Oncology, ¹¹Neurosurgery, Hospital de São Marcos, Braga; Departments of ¹²Neuropathology, ¹³Neurosurgery, Hospital Santo António, Porto; ¹⁴IPATIMUP, Porto; ¹⁵Medical Faculty, University of Porto, Porto; ¹⁶Institute CUF, Porto, Portugal.

Prognostic value of *MGMT* promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: A Portuguese multicentre study

BRUNO M. COSTA¹, CLÁUDIA CAEIRO², INÊS GUIMARÃES¹, OLGA MARTINHO¹, TERESA JARAQUEMADA², ISABEL AUGUSTO², LÍGIA CASTRO³, LÍGIA OSÓRIO⁴, PAULO LINHARES⁵, MRINALINI HONAVAR⁶, MÁRIO RESENDE⁷, FÁTIMA BRAGA⁸, ANA SILVA⁹, FERNANDO PARDAL⁹, JÚLIA AMORIM¹⁰, RUI NABIÇO¹⁰, RUI ALMEIDA¹¹, CARLOS ALEGRIA¹¹, MANUEL PIRES¹², CÉLIA PINHEIRO¹³, ERNESTO CARVALHO¹³, JOSÉ M. LOPES^{3,14,15}, PAULO COSTA¹⁶, MARGARIDA DAMASCENO² and RUI M. REIS¹

¹Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, Braga; Departments of ²Oncology, ³Pathology, ⁴Radiology, ⁵Neurosurgery, Hospital S. João, Porto; Departments of ⁶Pathology, ⁷Neurosurgery, ⁸Oncology, Hospital Pedro Hispano, Matosinhos; Departments of ⁹Pathology, ¹⁰Oncology, ¹¹Neurosurgery, Hospital de São Marcos, Braga; Departments of ¹²Neuropathology, ¹³Neurosurgery, Hospital Santo António, Porto; ¹⁴IPATIMUP, Porto; ¹⁵Medical Faculty, University of Porto, Porto; ¹⁶Institute CUF, Porto, Portugal

Received December 14, 2009; Accepted January 22, 2010

DOI: 10.3892/or_00000808

Abstract. Glioblastoma (GBM) is the most common and aggressive primary brain tumor. The identification of novel molecular prognostic markers of GBM has recently been an area of great interest in neuro-oncology. The methylation status of the *MGMT* gene promoter is currently a promising molecular prognostic marker, but some controversial data have precluded its clinical use. We analyzed *MGMT* methylation by methylation-specific PCR in 90 GBM patients from four Portuguese hospitals, uniformly treated with radiotherapy combined with concomitant and adjuvant temozolomide (Stupp protocol). The Kaplan-Meier method was used to construct survival curves, and the log-rank test and a Cox-regression model were used to analyze patient survival. The methylation status of *MGMT* was successfully determined in

89% (80/90) of the tumors. The frequency of tumoral *MGMT* promoter methylation was 47.5%. The median overall survival (OS) was 16 months (95% CI 12.2-19.8) and 13 months (95% CI 13.3-18.7) for patients whose tumors had a methylated or unmethylated *MGMT*, respectively. Univariate and multivariate analyses did not show any statistically significant association between *MGMT* methylation status and patient OS ($P=0.583$ by the log-rank test; $P=0.617$ by the Cox-regression test) or progression-free survival ($P=0.775$ by the log-rank test; $P=0.691$ by the Cox-regression test). None of the patient clinical features were significantly correlated with survival. This is the first study to report the frequency of *MGMT* methylation among Portuguese GBM patients. Our data did not show statistically significant associations between *MGMT* promoter methylation and the outcome of GBM patients treated with temozolomide. Additional robust prospective studies are warranted to clarify whether the *MGMT* status should be used in clinical decisions.

Correspondence to: Dr Rui Manuel Reis, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
E-mail: rreis@icsaude.uminho.pt

Abbreviations: GBM, glioblastoma multiforme; TMZ, temozolomide; OS, overall survival; PFS, progression-free survival; KPS, Karnofsky performance status; EORTC, European Organisation for Research and Treatment of Cancer; NCIC, National Cancer Institute of Canada

Key words: glioblastoma, prognosis, *MGMT* methylation, temozolomide, chemoradiation

Introduction

Tumors of the central nervous system account for a small percentage of all types of human tumors (1), but encompass a wide variety of distinct disease entities (2). Data from the GLOBOCAN 2002 database report an incidence of brain and central nervous system tumors in Portugal of ~422 cases per year in males and ~362 cases per year in females (3). Gliomas are the most common primary brain tumors (2,4) and encompass a wide variety of tumors thought to originate either from stem cells, glial precursor cells or glial differentiated cells. Astrocytomas are the major histological subtype, accounting for ~75% of all gliomas (4), of which GBM

(WHO grade IV) is by far the most common and malignant. The highly infiltrative nature coupled with the high proliferative potential causes this tumor to be particularly devastating, for which no curative therapies are currently available (5,6). Two main subtypes of GBM can be distinguished based on their clinical presentation: the most common subtype, primary (or *de novo*) GBM, develops without the presence of any precursor neoplastic lesion and manifests after a short clinical history (usually less than 3 months); secondary GBMs are much rarer and develop from lower grade tumors (2,7).

Despite recent improvements in therapeutic approaches, treatment still remains mostly palliative, and GBM patients usually present an extremely poor prognosis. The median survival is typically reported to be ~12 months in clinical trials (2), but a large population-based study in 2004 reported a median survival for these patients of ~5 months, with only ~18% and ~3% of the patients surviving over 1 and 2 years, respectively (8). The current paradigm of therapy for patients with newly diagnosed GBM includes surgical resection (when feasible), radiotherapy and chemotherapy. A phase III trial by Stupp *et al* clearly showed that GBM patients treated with radiotherapy combined with concomitant and adjuvant temozolomide (TMZ, an orally administered alkylating agent), also known as TMZ-based chemoradiation, had an increased overall survival (OS) compared with patients treated with radiotherapy alone (9), establishing a new standard in the management of these tumors. Despite a significant improvement, it is important to note that this treatment resulted in a small difference in overall survival. In order to improve the clinical outcome of these patients, it is widely acknowledged that the ideal treatment of GBMs must be individualized, based on the particular features of the tumor. Indeed, the prognosis of these patients is quite variable and unpredictable. Some of the most well-established prognostic markers in GBM include patient clinical features, such as age, Karnofsky performance status (KPS) (10) and extent of tumor resection; however, these markers do not satisfactorily predict patient outcome (11). One of the major goals of current neuro-oncology research is to identify robust and clinically relevant molecular markers that can add value to those more classic clinical prognostic factors.

MGMT (O-6-methylguanine-DNA methyltransferase), a gene located on chromosome 10q26, encodes a DNA-repair enzyme that has been shown to contribute to the chemoresistance of GBM cells to alkylating agents (12). Specifically, a landmark study by Hegi *et al* showed that GBM patients whose tumors had a methylated *MGMT* promoter presented a significantly longer median OS (21.7 months) and 2-year survival rate (46%) when treated with TMZ-based chemoradiation compared with patients without *MGMT* promoter methylation who were treated similarly (median survival of 12.7 months and 2-year survival rate of 13.8%) (13). Additionally, in the data set of Hegi *et al*, *MGMT* promoter methylation was an independent favorable prognostic factor, irrespective of treatment. Due to its potential prognostic value, the assessment of *MGMT* methylation status is currently a common practice in clinical trials involving GBM patients (14). Despite these striking findings, there is a significant body of controversial data surrounding the reproducibility of these results, questioning the true implication of *MGMT* as a

Table I. Clinicopathological features of glioblastoma patients treated with temozolomide-based chemoradiation (n=90).

Male/female ratio	1.9
Age, years (median $\pm$ SD)	56 $\pm$ 11
Karnofsky performance score	
$\geq$ 80 (n)	48
<80 (n)	42
Extent of resection	
Total or subtotal (n)	81
Biopsy (n)	9

n, number of patients.

GBM prognostic marker and/or a specific predictor of TMZ-based chemotherapy (15-17). In this context, we aimed to assess the frequency and clarify the prognostic capacity of *MGMT* promoter methylation in a set of Portuguese GBM patients uniformly treated with TMZ-based chemoradiation.

Materials and methods

Human tumor samples. Human tumor samples were obtained from primary GBM patients newly diagnosed according to the WHO criteria (2) and surgically resected between 2004 and 2007 at 4 hospitals in northern Portugal: Hospital São João (n=36), Hospital Pedro Hispano (n=33), Hospital Santo António (n=11) and Hospital São Marcos (n=10). All patients underwent radiotherapy plus continuous concomitant TMZ after surgery, followed by maintenance cycles of TMZ, according to the Stupp protocol (9). Extension of tumor resection was assessed by the neurosurgeon and by post-operative magnetic resonance imaging (MRI) and were classified as gross resection (total or subtotal) or biopsy. The clinicopathological features are summarized in Table I. All procedures followed in this study were in accordance with institutional ethical standards, and the biological samples were unlinked and unidentified from their donors. Follow-up data were available for all patients as of July 2008, and were collected through direct interview with patients or their relatives and by review of in-hospital patient files.

DNA isolation. DNA was isolated by macrodissection from 10- μ m sections of formalin-fixed paraffin-embedded tumor tissue samples, avoiding the harvesting of surrounding normal brain tissue by comparing each slide with the corresponding hematoxylin and eosin (H&E)-stained slide (marked for the area of tumoral tissue) (18). The recovered tissues were processed with the QIAamp® DNA Micro Kit (Qiagen) following the manufacturer's instructions. DNA quality control and yield were assessed by spectrophotometry using Nanodrop™.

DNA bisulfite treatment and *MGMT* methylation-specific PCR analysis. Genomic DNA was subjected to bisulfite treatment using the EZ DNA Methylation-Gold™ Kit (Zymo

Table II. Associations between clinical features or status of tumoral *MGMT* promoter methylation and prognosis of GBM patients, assessed by univariate (log-rank test) and multivariate (Cox-regression) analyses.

	Overall survival			Progression-free survival		
	Median (95% CI) ^a	P-value (Log-rank)	P-value (Cox)	Median (95% CI) ^a	P-value (Log-rank)	P-value (Cox)
Gender						
Males	14 (10.6-17.4)			9 (6.9-11.1)		
Females	14 (11.6-16.4)	0.436	0.807	9 (5.9-12.1)	0.864	0.990
Age ^b						
>56	13 (9.5-16.5)			9 (6.9-11.1)		
≤56	14 (11.0-17.0)	0.978	0.951	10 (8.0-12.0)	0.754	0.431
KPS						
≥80	16 (14.2-17.8)			10 (7.2-12.8)		
<80	12 (9.8-14.2)	0.165	0.311	8 (5.7-10.3)	0.096	0.125
Extent of resection						
Total or subtotal	13 (10.4-15.6)			9 (7.5-10.5)		
Biopsy	16 (13.3-18.7)	0.458	0.481	6 (1.1-10.9)	0.942	0.930
<i>MGMT</i> status						
Methylated	16 (12.2-19.8)			9 (5.0-13.0)		
Unmethylated	13 (11.1-14.9)	0.583	0.617	10 (8.1-12.0)	0.775	0.691

^aMedian survival and 95% confidence intervals, in months. ^bPatient age was used as a continuous variable for the Cox-regression model. KPS, Karnofsky performance status.

Research) following the manufacturer's instructions. The promoter *MGMT* methylation-specific polymerase chain reactions (MSP) were performed using a two-step nested approach, and the results were confirmed by one-step MSP in a subset of tumors as previously described (13,19). Peripheral blood DNA from tumor-free controls and CpGenome™ Universal Methylated DNA (Chemicon International) were used as *MGMT* unmethylated and methylated controls, respectively. The PCR products were resolved on 4% low-melting point agarose gels. Analysis of MSP data was performed by investigators who were blind to the clinical data.

Statistical analyses. The Kaplan-Meier method was used to estimate OS and progression-free survival (PFS), and the log-rank test was used to assess the differences. OS was measured from the time of surgical resection to patient death, or the last date when the patient was known to be alive. PFS time was defined as the time from surgical resection to the time of demonstrated tumor growth on follow-up imaging, or evidence of neurological decline. Multivariate survival analyses by use of Cox proportional hazards models (backward selection) were performed to adjust for the effects of potential confounding factors, including patient age (used as a continuous variable), gender, KPS and extent of tumor resection. All statistical tests were two-sided, and significance was considered at values of $P < 0.05$. Data analysis was performed using SPSS 16.0 software (SPSS, Inc.).

Results

Patient clinical features which may have an effect on prognosis, such as gender, age at diagnosis, KPS and extent of tumor resection, are summarized in Table I. The tumoral *MGMT* methylation status was successfully determined by MSP in 80 tumor samples (89%), of which 38 GBMs (47.5%) had a methylated *MGMT* promoter. Fig. 1 presents a typical MSP analysis of the methylation status of the *MGMT* promoter.

Considering the whole tumor set, independently of clinical features or *MGMT* methylation status, the median OS and PFS were 14 months (95% CI 11.5-16.5) and 9 months (95% CI 7.5-10.5), respectively. The overall 2-year survival rate was 16.7%. Table II summarizes the median OS and median PFS of patients based on clinicopathological features (gender, age, KPS and extent of tumor resection) and *MGMT* methylation status. By using the log-rank test, no statistically significant associations were detected between each individual variable and GBM patient OS or PFS ($P > 0.05$) (Table II). Similarly, a multivariate Cox proportional model did not show any statistically significant correlation between the studied variables and GBM patient outcome ($P > 0.05$) (Table II). Nevertheless, patients whose tumors had a methylated *MGMT* promoter showed a slightly improved median OS of 16 months (95% CI 12.2-19.8) as compared to patients with an unmethylated *MGMT* promoter (13 months, 95% CI 11.1-14.9; Fig. 2), but the differences were not statistically significant ($P = 0.583$ by the univariate test; $P = 0.617$ by the multivariate

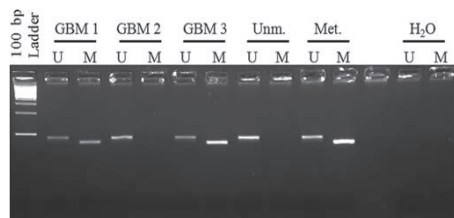


Figure 1. Methylation-specific PCR (MSP) analyses of the *MGMT* promoter in glioblastoma tumor tissue from three patients (GBM 1, GBM 2 and GBM 3). MSP control reactions consisted of blood-extracted DNA from a cancer-free individual for use as the unmethylated DNA control (Unm.), and a CpGenome Universal Methylated DNA as the methylated DNA control (Met.). Note the presence of bands in both the unmethylated (U, 93 bp) and methylated (M, 81 bp) lanes for glioblastoma samples 1 and 3, reflecting a methylated *MGMT* promoter. The lack of a band in the lane corresponding to methylation-specific primers for glioblastoma sample 2 reflects the absence of *MGMT* promoter methylation. PCR reactions in the absence of DNA (H₂O) were performed as negative controls for both the unmethylated and methylated reactions.

model; Table II). The median PFS was closely similar between patients whose tumors had methylated (9 months, 95% CI 5.0-13.0) and unmethylated (10 months, 95% CI 8.1-12.0) *MGMT* promoter ($P=0.775$ by the log-rank test; $P=0.691$ by the Cox model; Table II). The 2-year survival rate was 15.8% and 16.7% in patients with methylated and unmethylated *MGMT*, respectively ($P=0.915$).

Discussion

GBM is a particularly devastating disease as no curative therapies are available, and very few well-established prognostic factors have been identified. Many recent efforts in the field of neuro-oncology are directed to developing more efficient therapies, but only a small fraction of patients experience significant clinical benefit and prolonged survival. Despite the variability of the clinical responses, the majority of patients with GBM are presently treated in a uniform standardized way, following a 'one fits all' therapeutic approach, regardless of the individual molecular characteristics of each tumor that most likely affect patient prognosis. Consequently, many patients display minor responses and major therapy-related toxicities.

The recent introduction of radiotherapy plus concomitant and adjuvant TMZ treatment for GBM has led to a small but significant improvement in patient outcome (9); however, the responses are still very poor and unpredictable. While the EORTC-NCIC trial by Hegi *et al* (13) implicated the status of *MGMT* promoter methylation as a biomarker of GBM patient response to TMZ, all GBM patients continue to be treated with TMZ, regardless of their *MGMT* status (13). To note, the conclusions of Hegi *et al* were somewhat confounded and questioned due to several limitations of the study. First, the studies were performed retrospectively on a subset of patients from whom adequate tumor tissue was available. Second, although the difference in survival based on *MGMT* methylation status was highly significant, more than half of

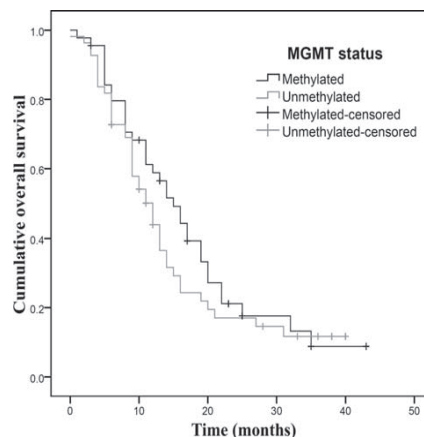


Figure 2. Kaplan-Meier analyses of the influence of *MGMT* promoter methylation on overall survival of glioblastoma patients treated with temozolomide-based chemoradiation. Patients whose tumors present a methylated *MGMT* promoter (black curve) show a trend of improved overall survival than patients whose tumors have an unmethylated *MGMT* promoter (grey curve), but the differences did not reach statistical significance ($P=0.583$ by the log-rank test; see text for details).

the methylated *MGMT* patients (with a predictably favorable outcome) did not survive for 2 years, while ~15% of the patients with unfavorable unmethylated *MGMT* did survive over 2 years. Third, *MGMT* methylation was associated with improved outcomes both in the temozolomide-based chemoradiation and radiation-only arms, suggesting that it may partly be a prognostic marker associated with the nature of the tumor, rather than a truly therapy-specific predictive marker. Lastly, many of the patients in the radiation-only arm also received TMZ at recurrence. Regardless, this was a landmark study suggesting that methylation of *MGMT* is both a prognostic marker and a specific marker of response to TMZ in GBM patients. Indeed, the conclusions of Hegi *et al* were recently corroborated by a 5-year analysis of the EORTC-NCIC trial, where Stupp *et al* (20) confirmed that the methylation of the *MGMT* promoter is associated with better outcome and benefit from TMZ chemotherapy. The first study implicating a correlation between *MGMT* levels and survival of malignant glioma patients dates back to 1998, when Jaeckle *et al* showed that patients treated with BCNU (a nitrosourea used as a chemotherapeutic agent) whose tumors had low or undetectable levels of *MGMT* had an improved survival as compared to those whose tumors had detectable *MGMT* activity (21). Soon after, Esteller *et al* reported for the first time that methylation of the *MGMT* gene promoter led to loss of *MGMT* expression in human tumors *in vivo* (22). In 2000, Esteller *et al* found that a methylated *MGMT* promoter in malignant glioma was associated with improved overall and PFS, independently of patient age and KPS (12). Mechanistically, the ability of functional *MGMT* protein to remove the cytotoxic chemotherapy-induced alkyl groups from the O⁶-guanine of DNA, and thus prevent killing of tumor cells, supported the clinical

Table III. Prognostic significance of *MGMT* methylation in glioblastoma reported in the literature.

Author (Reference)	Year	n	Treatment	Effect of <i>MGMT</i> methylation on survival ^a	
				PFS	OS
Esteller, <i>et al</i> (12)	2000	29	BCNU	Yes	Yes
Balana, <i>et al</i> (16)	2003	21	TMZ + cisplatin (n=7)	NS	ND
			BCNU (n=7)	Yes	ND
			No treatment (n=3)	ND	ND
Paz, <i>et al</i> (26)	2004	51	BCNU or CCNU or TMZ	NS	NS
Hegi, <i>et al</i> (27)	2004	38	Concomitant + adjuvant TMZ	ND	Yes
Blanc, <i>et al</i> (28)	2004	44	BCNU or fotemustine (n=2); radiation-only (n=14);		
			Radiation + chemotherapy (BCNU or fotemustine) (n=22);		
			No treatment (n=6)	NS	NS
Kamiryo, <i>et al</i> (17)	2004	74	ACNU	NS	NS
Watanabe, <i>et al</i> (29)	2004	29	IAR regimen	Yes	Yes
Hegi, <i>et al</i> ^b (13)	2005	100	Radiotherapy only	ND	Yes
			Concomitant + adjuvant TMZ	Yes	Yes
Brandes, <i>et al</i> (42)	2006	22 (recurrent GBMs)	TMZ after recurrence	NS	NS
Herrlinger, <i>et al</i> (31)	2006	19	CCNU + TMZ	Yes	Yes
Piccirilli, <i>et al</i> (41)	2006	22 (≥80 years)	Chemotherapy not specified	ND	Yes
Wick, <i>et al</i> (30)	2007	36	Alternating weekly TMZ	NS	NS
Eoli, <i>et al</i> (33)	2007	86	1st line cisplatin + BCNU;		
			PCV or TMZ after recurrence	Yes	Yes
			Concomitant + adjuvant nitrosureas	ND	Yes
Criniere, <i>et al</i> (32)	2007	39	Adjuvant nitrosureas	ND	NS
			Radiotherapy only	ND	NS
			TMZ (n=7) or etoposide (n=3)	Yes	Yes
Donson, <i>et al</i> (40)	2007	10 (pediatric GBM)	Concomitant + adjuvant TMZ	Yes	Yes
Brandes, <i>et al</i> (45)	2008	103	Gliadel wafers	ND	Yes
Smith, <i>et al</i> (48)	2008	27	Concomitant + adjuvant TMZ	Yes	Yes
Murat, <i>et al</i> (39)	2008	42	Concomitant + adjuvant TMZ	NS	NS
Sijben, <i>et al</i> (50)	2008	29 (≥65 years)	Concomitant + adjuvant TMZ	ND	Yes
Gorlia, <i>et al</i> ^b (35)	2008	106	Concomitant + adjuvant TMZ	Yes	Yes
Dunn, <i>et al</i> (44)	2009	109 ^c	Concomitant + adjuvant TMZ	Yes	Yes
Brandes, <i>et al</i> (23)	2009	37 (≥65 years)	Concomitant + adjuvant TMZ	NS	NS
Clarke, <i>et al</i> (46)	2009	48	TMZ (metronomic versus dose-dense)	ND	NS
Wemmert, <i>et al</i> (36)	2009	27	Radiotherapy only	ND	Yes
Stupp, <i>et al</i> ^b (20)	2009	100	Concomitant + adjuvant TMZ	Yes	Yes
			CCNU + TMZ	Yes	Yes
Glas, <i>et al</i> (38)	2009	23	ACNU + cisplatin	ND	NS
Park, <i>et al</i> (47)	2009	48	Erlotinib + TMZ	ND	Yes
Prados, <i>et al</i> (37)	2009	44	TMZ after recurrence	NS	NS
Sadones, <i>et al</i> (49)	2009	22	Concomitant + adjuvant TMZ	NS	NS
Schaich, <i>et al</i> (43)	2009	61	ACNU	Yes	Yes
Sonoda, <i>et al</i> (25)	2009	30 ^d	Not specified	ND	Yes/No ^e
Mellai, <i>et al</i> (24)	2009	101	Carmustine wafers	Yes	Yes
Metellus, <i>et al</i> (51)	2009	19 (recurrent GBMs)	Concomitant + adjuvant TMZ and talampanel	Yes	Yes
Grossman, <i>et al</i> (52)	2009	29	TMZ	ND	NS
Martinez, <i>et al</i> (53)	2009	46 ^f	Not specified	ND	Yes ^g
Piperi, <i>et al</i> (54)	2009	17	Radiotherapy-only	NS	NS
Weller, <i>et al</i> (55)	2009	63	Concomitant + adjuvant TMZ	Yes	Yes

n, number of patients; PFS, progression-free survival; OS, overall survival; NS, not significant; ND, not determined; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (nimustine); BCNU, 1,3-bis-(chloro-ethyl)-1-nitrosourea (carmustine, Gliadel); CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); IAR, IFN- β , ACNU and radiotherapy; PCV, procarbazine, lomustine and vincristine; TMZ, temozolomide. ^aOnly statistically significant results ($P < 0.05$) are considered. ^bThese studies analyzed the same tumor set from the EORTC-NCIC trial. ^c*MGMT* promoter was considered methylated by pyrosequencing when $\geq 9\%$ of CpG dinucleotides were methylated. ^dThe set of 30 GBM patients in this study compared long-term and short-term survivors only. ^eStatistically significant by univariate analysis, but not significant by multivariate analysis. ^f*MGMT* methylation status was determined by a microarray-based DNA methylation analysis. ^gPatients with a methylated *MGMT* promoter had a significantly worse prognosis than unmethylated cases.

finding that lack of *MGMT* expression was associated with improved outcomes. Whether this molecular marker should ultimately direct the treatment of newly diagnosed patients is still a fundamental and controversial question. Much of the controversy surrounding the prognostic value of *MGMT* is partly due to studies including very heterogeneous groups of patients with different glioma subtypes and who underwent different treatment regimens. In addition, not all studies analyzed the *MGMT* status at the level of DNA methylation in the promoter region by methylation-specific polymerase chain reaction (MSP); some studies focused on mRNA expression by RT-PCR and protein levels by immunohistochemistry and quantitative immunofluorescence (15). Nevertheless, contradictory results are also reported even in studies that evaluated the influence of *MGMT* promoter methylation on the prognosis of patients with GBM (Table III) (12,13,16,17,20,23-55).

In our study, we attempted to clarify whether *MGMT* methylation is a biomarker of clinical outcome in GBM patients treated with the recently introduced TMZ-based chemoradiation protocol and how it compares to the prognostic value of classic clinicopathological factors. By studying a set of 90 GBM patients from northern Portugal, we were unable to detect any significant correlations between patient clinical features, *MGMT* methylation status and prognosis. Other studies have also failed to show such correlations (Table III). To note, the frequency of *MGMT* promoter methylation in our data set is similar to the previously reported frequencies (13,23,30,33,35,55). Additionally, the median OS and median PFS in our set of GBM patients are in line with other recently published studies (20,23,55). Some possible limitations of our study include the relatively small sample size, which may be a limiting factor in achieving statistical significance, and the fact that we analyzed a multicenter tumor set collected from 4 independent institutions. Nevertheless, our sample size compares favorably with other similar studies published in the literature (Table III); additionally, our population-based study is likely to closely illustrate the difficulties in identifying significant determinants of patient survival, as this type of study is typically less controlled and more heterogeneous than well-designed prospective clinical trials. Obviously, the argument that *MGMT* methylation does not predict GBM patient response to this therapeutic regimen is still equally valid. It is also relevant to stress that the most appropriate method to assess *MGMT* status in gliomas is quite controversial. Validated and commercially available *MGMT* methylation assays have not yet been approved for clinical use and may indeed be technically challenging. In spite of a recent report arguing in favor of the feasibility and reliability of nested, gel-based methylation-specific PCR (MSP) analysis, suggesting it could be routinely implemented in the clinical setting (56), the use of MSP is often considered not to be so straightforward (57-60). Its use has raised some concerns due to inter- and intra-test variability and sensitivity and specificity issues (57,59). One recent report argues that a quantitative MSP test for *MGMT* methylation is more specific than conventional gel-based MSP (61). Furthermore, *MGMT* silencing can occur through methylation of specific cytosines within the CpG island (62); thus, it is important to determine

which CpG dinucleotides are the most predictive of potential *MGMT* silencing. A recent study using pyrosequencing methylation analysis argues that a new set of CpG dinucleotides in the *MGMT* promoter CpG island is more robust in predicting gene silencing than those classically tested by the MSP assay (63). Conceptually, while a methylated *MGMT* promoter precludes gene expression, the absence of such methylation does not necessarily translate into activation of gene expression, as specific transcription factors [e.g., Sp1 (64) and TP53 (65)], permissive chromatin states (64,66-69), and absence of negative transcriptional regulators (e.g., IFN- β) are also required. Additionally, it is reasonable to assume that, given the heterogeneity of *MGMT* expression within individual tumors, a fraction of the cells within the entire tumor load may lack *MGMT* expression, despite an unmethylated *MGMT* status, and may therefore be sensitive to temozolomide. A recent study also suggests that variation in *MGMT* promoter methylation can occur within the same tumor after treatment (70), stressing that clinical decisions based on *MGMT* require caution. Despite these considerations as well as the consensual belief that additional molecular markers of GBM patient outcome are important in determining the tumor response to therapy, if the relevance of *MGMT* promoter methylation status in GBM is corroborated by an ongoing phase III large study by the Radiation Therapy Oncology Group (RTOG 0525), patients with unmethylated *MGMT* promoter may be selected for alternative treatment options in the future. Potential alternative strategies to overcome *MGMT*-mediated chemoresistance include: (i) use of temozolomide together with *MGMT*-inactivating drugs (71,72) such as O⁶-benzylguanine (73,74) or inhibitors of other DNA repair enzymes; and (ii) use of dose-intensive temozolomide regimens (71) which deplete *MGMT* levels more rapidly than lower doses (75).

In conclusion, our study is the first to report the frequency of tumoral *MGMT* promoter methylation among Portuguese GBM patients and to analyze the correlations between clinical features, *MGMT* status and outcome in a set of patients uniformly treated with concomitant and adjuvant TMZ chemoradiation. In light of our data, together with the controversies reported in the literature, further studies are warranted to clarify the clinical prognostic relevance of *MGMT* methylation in GBM.

Acknowledgements

This project was sponsored, in part, by Schering-Plough Farma (Portugal). B.M.C. and O.M. are recipients of fellowships from the Portuguese Science and Technology Foundation (SFRH/BPD/33612/2009 and SFRH/BD/36463/2007). The funding institutions had no role in the study design, data collection and analysis, interpretation of the results, the preparation of the manuscript, or the decision to submit the manuscript for publication.

References

1. Espey DK, Wu XC, Swan J, *et al*: Annual report to the nation on the status of cancer, 1975-2004, featuring cancer in American Indians and Alaska Natives. *Cancer* 110: 2119-2152, 2007.

2. Louis DN, Ohgaki H, Wiestler OD, *et al*: The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 114: 97-109, 2007.
3. Parkin DM, Bray F, Ferlay J, and Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74-108, 2005.
4. CBRTRUS. Supplement Report: Primary Brain Tumors in the United States. Central Brain Tumor Registry of the United States, Hinsdale, IL, 2008
5. Franceschi E, Tosoni A, Bartolini S, Mazzocchi V, Fioravanti A, and Brandes AA: Treatment options for recurrent glioblastoma: pitfalls and future trends. *Expert Rev Anticancer Ther* 9: 613-619, 2009.
6. Reardon DA, Rich JN, Friedman HS, and Bigner DD: Recent advances in the treatment of malignant astrocytoma. *J Clin Oncol* 24: 1253-1265, 2006.
7. Ohgaki H and Kleihues P: Genetic pathways to primary and secondary glioblastoma. *Am J Pathol* 170: 1445-1453, 2007.
8. Ohgaki H, Dessen P, Jourde B, *et al*: Genetic pathways to glioblastoma: a population-based study. *Cancer Res* 64: 6892-6899, 2004.
9. Stupp R, Mason WP, van den Bent MJ, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-996, 2005.
10. Curran WJ Jr, Scott CB, Horton J, *et al*: Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. *J Natl Cancer Inst* 85: 704-710, 1993.
11. Rivera AL, Pelloski CE, Sulman E and Aldape K: Prognostic and predictive markers in glioma and other neuroepithelial tumors. *Curr Probl Cancer* 32: 97-123, 2008.
12. Esteller M, Garcia-Foncillas J, Andion E, *et al*: Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. *N Engl J Med* 343: 1350-1354, 2000.
13. Hegi ME, Diserens AC, Gorlia T, *et al*: MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 352: 997-1003, 2005.
14. Clinical Trials with Glioblastoma Patients. <http://clinicaltrials.gov/2009>. January 7. Available from: URL: <http://clinicaltrials.gov/ct2/results?term=glioblastoma>.
15. Brelvi M, Tortosa A, Verger E, *et al*: Prognostic significance of O⁶-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clin Cancer Res* 11: 5167-5174, 2005.
16. Balana C, Ramirez JL, Taron M, *et al*: O⁶-methyl-guanine-DNA methyltransferase methylation in serum and tumor DNA predicts response to 1,3-bis(2-chloroethyl)-1-nitrosourea but not to temozolomide plus cisplatin in glioblastoma multiforme. *Clin Cancer Res* 9: 1461-1468, 2003.
17. Kamiyori T, Tada K, Shiraishi S, Shinjima N, Kochi M and Ushio Y: Correlation between promoter hypermethylation of the O⁶-methylguanine-deoxyribonucleic acid methyltransferase gene and prognosis in patients with high-grade astrocytic tumors treated with surgery, radiotherapy, and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea-based chemotherapy. *Neurosurgery* 54: 349-357, 2004.
18. Basto D, Trovisco V, Lopes JM, *et al*: Mutation analysis of B-RAF gene in human gliomas. *Acta Neuropathol* 109: 207-210, 2005.
19. Palmisano WA, Divine KK, Saccomanno G, *et al*: Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res* 60: 5954-5958, 2000.
20. Stupp R, Hegi ME, Mason WP, *et al*: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 10: 459-466, 2009.
21. Jaeckle KA, Eyre HJ, Townsend JJ, *et al*: Correlation of tumor O⁶ methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bis-chloroethyl-nitrosourea: a Southwest Oncology Group study. *J Clin Oncol* 16: 3310-3315, 1998.
22. Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG: Inactivation of the DNA repair gene O⁶-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 59: 793-797, 1999.
23. Brandes AA, Franceschi E, Tosoni A, *et al*: Temozolomide concomitant and adjuvant to radiotherapy in elderly patients with glioblastoma: correlation with MGMT promoter methylation status. *Cancer* 115: 3512-3518, 2009.
24. Mellai M, Caldera V, Annovazzi L, *et al*: MGMT promoter hypermethylation in a series of 104 glioblastomas. *Cancer Genomics Proteomics* 6: 219-227, 2009.
25. Sonoda Y, Kumabe T, Watanabe M, *et al*: Long-term survivors of glioblastoma: clinical features and molecular analysis. *Acta Neurochir* 151: 1349-1358, 2009.
26. Paz MF, Yaya-Tur R, Rojas-Marcos I, *et al*: CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. *Clin Cancer Res* 10: 4933-4938, 2004.
27. Hegi ME, Diserens AC, Godard S, *et al*: Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. *Clin Cancer Res* 10: 1871-1874, 2004.
28. Blanc JL, Wager M, Guilhot J, *et al*: Correlation of clinical features and methylation status of MGMT gene promoter in glioblastomas. *J Neurooncol* 68: 275-283, 2004.
29. Watanabe T, Katayama Y, Komine C, *et al*: O⁶-methylguanine-DNA methyltransferase methylation and TP53 mutation in malignant astrocytomas and their relationships with clinical course. *Int J Cancer* 113: 581-587, 2005.
30. Wick A, Felsberg J, Steinbach JP, *et al*: Efficacy and tolerability of temozolomide in an alternating weekly regimen in patients with recurrent glioma. *J Clin Oncol* 25: 3357-3361, 2007.
31. Herrlinger U, Rieger J, Koch D, *et al*: Phase II trial of Lomustine plus temozolomide chemotherapy in addition to radiotherapy in newly diagnosed glioblastoma: UKT-03. *J Clin Oncol* 24: 4412-4417, 2006.
32. Criniere E, Kaloshi G, Laigle-Donadey F, *et al*: MGMT prognostic impact on glioblastoma is dependent on therapeutic modalities. *J Neurooncol* 83: 173-179, 2007.
33. Eoli M, Menghi F, Bruzzone MG, *et al*: Methylation of O⁶-methylguanine DNA methyltransferase and loss of heterozygosity on 19q and/or 17p are overlapping features of secondary glioblastomas with prolonged survival. *Clin Cancer Res* 13: 2606-2613, 2007.
34. Brandes AA, Tosoni A, Franceschi E, *et al*: Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation with MGMT promoter methylation status. *J Clin Oncol* 27: 1275-1279, 2009.
35. Gorlia T, van den Bent MJ, Hegi ME, *et al*: Nomograms for predicting survival of patients with newly diagnosed glioblastoma: prognostic factor analysis of EORTC and NCIC trial 26981-22981/CE.3. *Lancet Oncol* 9: 29-38, 2008.
36. Wemmer S, Bettscheider M, Alt S, *et al*: p15 promoter methylation - A novel prognostic marker in glioblastoma patients. *Int J Oncol* 34: 1743-1748, 2009.
37. Prados MD, Chang SM, Butowski N, *et al*: Phase II study of erlotinib plus temozolomide during and after radiation therapy in patients with newly diagnosed glioblastoma multiforme or gliosarcoma. *J Clin Oncol* 27: 579-584, 2009.
38. Glas M, Hapold C, Rieger J, *et al*: Long-term survival of patients with glioblastoma treated with radiotherapy and lomustine plus temozolomide. *J Clin Oncol* 27: 1257-1261, 2009.
39. Murat A, Migliauacca E, Gorlia T, *et al*: Stem cell-related 'self-renewal' signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. *J Clin Oncol* 26: 3015-3024, 2008.
40. Donson AM, Addo-Yobo SO, Handler MH, Gore L and Foreman NK: MGMT promoter methylation correlates with survival benefit and sensitivity to temozolomide in pediatric glioblastoma. *Pediatr Blood Cancer* 48: 403-407, 2007.
41. Piccirilli M, Bistazzoni S, Gagliardi FM, *et al*: Treatment of glioblastoma multiforme in elderly patients. Clinico-therapeutic remarks in 22 patients older than 80 years. *Tumori* 92: 98-103, 2006.
42. Brandes AA, Tosoni A, Cavallo G, *et al*: Temozolomide 3 weeks on and 1 week off as first-line therapy for recurrent glioblastoma: phase II study from Gruppo Italiano Cooperativo di Neuro-oncologia (GICNO). *Br J Cancer* 95: 1155-1160, 2006.
43. Schaich M, Kestel L, Pfirrmann M, *et al*: A MDR1 (ABCB1) gene single nucleotide polymorphism predicts outcome of temozolomide treatment in glioblastoma patients. *Ann Oncol* 20: 175-181, 2009.
44. Dunn J, Baborie A, Alam F, *et al*: Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. *Br J Cancer* 101: 124-131, 2009.

45. Brandes AA, Franceschi E, Tosoni A, *et al*: MGMT promoter methylation status can predict the incidence and outcome of pseudoprogression after concomitant radiochemotherapy in newly diagnosed glioblastoma patients. *J Clin Oncol* 26: 2192-2197, 2008.
46. Clarke JL, Iwamoto FM, Sul J, *et al*: Randomized phase II trial of chemoradiotherapy followed by either dose-dense or metronomic temozolomide for newly diagnosed glioblastoma. *J Clin Oncol* 27: 3861-3867, 2009.
47. Park CK, Park SH, Lee SH, *et al*: Methylation status of the MGMT gene promoter fails to predict the clinical outcome of glioblastoma patients treated with ACNU plus cisplatin. *Neuropathology* 29: 443-449, 2009.
48. Smith KA, Ashby LS, Gonzalez F, *et al*: Prospective trial of gross-total resection with Gliadel wafers followed by early post-operative gamma knife radiosurgery and conformal fractionated radiotherapy as the initial treatment for patients with radiographically suspected, newly diagnosed glioblastoma multiforme. *J Neurosurg* 109 (Suppl): 106-117, 2008.
49. Sadones J, Michotte A, Veld P, *et al*: MGMT promoter hypermethylation correlates with a survival benefit from temozolomide in patients with recurrent anaplastic astrocytoma but not glioblastoma. *Eur J Cancer* 45: 146-153, 2009.
50. Sijben AE, McIntyre JB, Roldan GB, *et al*: Toxicity from chemoradiotherapy in older patients with glioblastoma multiforme. *J Neurooncol* 89: 97-103, 2008.
51. Metellus P, Coulibaly B, Nanni I, *et al*: Prognostic impact of O(6)-methylguanine-DNA methyltransferase silencing in patients with recurrent glioblastoma multiforme who undergo surgery and carmustine wafer implantation: a prospective patient cohort. *Cancer* 115: 4783-4794, 2009.
52. Grossman SA, Ye X, Chamberlain M, *et al*: Talampanel with standard radiation and temozolomide in patients with newly diagnosed glioblastoma: a multicenter phase II trial. *J Clin Oncol* 27: 4155-4161, 2009.
53. Martinez R, Martin-Subero JI, Rohde V, *et al*: A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics* 4: 255-264, 2009.
54. Piperi C, Themistocleous MS, Papavassiliou GA, *et al*: High incidence of MGMT and RARbeta promoter methylation in primary glioblastomas: association with histopathological characteristics, inflammatory mediators and clinical outcome. *Mol Med* 16: 1-9, 2009.
55. Weller M, Felsberg J, Hartmann C, *et al*: Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: A prospective translational study of the German Glioma Network. *J Clin Oncol* 27: 5743-5750, 2009.
56. Yachi K, Watanabe T, Ohta T, *et al*: Relevance of MSP assay for the detection of MGMT promoter hypermethylation in glioblastomas. *Int J Oncol* 33: 469-475, 2008.
57. Shen L, Guo Y, Chen X, Ahmed S and Issa JP: Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. *Biotechniques* 42: 48, 50, 52, passim, 2007.
58. Kagan J, Srivastava S, Barker PE, Belinsky SA and Cairns P: Towards clinical application of methylated DNA sequences as cancer biomarkers: A Joint NCI's EDNR and NIST Workshop on Standards, Methods, Assays, Reagents and Tools. *Cancer Res* 67: 4545-4549, 2007.
59. Vlassenbroeck I, Califice S, Diserens AC, *et al*: Validation of real-time methylation-specific PCR to determine O⁶-methylguanine-DNA methyltransferase gene promoter methylation in glioma. *J Mol Diagn* 10: 332-337, 2008.
60. Kitange GJ, Carlson BL, Mladek AC, *et al*: Evaluation of MGMT promoter methylation status and correlation with temozolomide response in orthotopic glioblastoma xenograft model. *J Neurooncol* 92: 23-31, 2009.
61. Parrella P, la Torre A, Copetti M, *et al*: High specificity of quantitative methylation-specific PCR analysis for MGMT promoter hypermethylation detection in gliomas. *J Biomed Biotechnol* 2009: 531692, 2009.
62. Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS and Futscher BW: Methylation of discrete regions of the O⁶-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. *Mol Cell Biol* 17: 5612-5619, 1997.
63. Everhard S, Tost J, El Abdalaoui H, *et al*: Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro Oncol* 11: 348-356, 2009.
64. Costello JF, Futscher BW, Kroes RA and Pieper RO: Methylation-related chromatin structure is associated with exclusion of transcription factors from and suppressed expression of the O-6-methylguanine DNA methyltransferase gene in human glioma cell lines. *Mol Cell Biol* 14: 6515-6521, 1994.
65. Blough MD, Zlatescu MC and Cairncross JG: O⁶-methylguanine-DNA methyltransferase regulation by p53 in astrocytic cells. *Cancer Res* 67: 580-584, 2007.
66. Danam RP, Howell SR, Brent TP and Harris LC: Epigenetic regulation of O⁶-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins. *Mol Cancer Ther* 4: 61-69, 2005.
67. Nakagawachi T, Soejima H, Urano T, *et al*: Silencing effect of CpG island hypermethylation and histone modifications on O⁶-methylguanine-DNA methyltransferase (MGMT) gene expression in human cancer. *Oncogene* 22: 8835-8844, 2003.
68. Zhao W, Soejima H, Higashimoto K, *et al*: The essential role of histone H3 Lys9 di-methylation and MeCP2 binding in MGMT silencing with poor DNA methylation of the promoter CpG island. *J Biochem* 137: 431-440, 2005.
69. Natsume A, Ishii D, Wakabayashi T, *et al*: IFN-beta down-regulates the expression of DNA repair gene MGMT and sensitizes resistant glioma cells to temozolomide. *Cancer Res* 65: 7573-7579, 2005.
70. Parkinson JF, Wheeler HR, Clarkson A, *et al*: Variation of O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation in serial samples in glioblastoma. *J Neurooncol* 87: 71-78, 2008.
71. Wick W, Platten M and Weller M: New (alternative) temozolomide regimens for the treatment of glioma. *Neuro Oncol* 11: 69-79, 2009.
72. Friedman HS: Can O⁶-alkylguanine-DNA alkyltransferase depletion enhance alkylator activity in the clinic? *Clin Cancer Res* 6: 2967-2968, 2000.
73. Quinn JA, Desjardins A, Weingart J, *et al*: Phase I trial of temozolomide plus O⁶-benzylguanine for patients with recurrent or progressive malignant glioma. *J Clin Oncol* 23: 7178-7187, 2005.
74. Spiro TP, Gerson SL, Liu L, *et al*: O⁶-benzylguanine: a clinical trial establishing the biochemical modulatory dose in tumor tissue for alkyltransferase-directed DNA repair. *Cancer Res* 59: 2402-2410, 1999.
75. Tolcher AW, Gerson SL, Denis L, *et al*: Marked inactivation of O⁶-alkylguanine-DNA alkyltransferase activity with protracted temozolomide schedules. *Br J Cancer* 88: 1004-1011, 2003.

2 IMPACT OF EGFR GENETIC VARIANTS ON GLIOMA RISK AND PATIENT OUTCOME

Bruno M Costa,^{1,2} Marta Viana-Pereira,^{1,2} Ricardo Fernandes,^{1,2} Sandra Costa,^{1,2} Paulo Linhares,³ Rui Vaz,³ Céline Pinheiro,^{1,2} Jorge Lima,⁴ Paula Soares,^{4,5} Ana Silva,⁶ Fernando Pardal,⁶ Júlia Amorim,⁷ Rui Nabiço,⁷ Rui Almeida,⁸ Carlos Alegria,⁸ Manuel M Pires,⁹ Célia Pinheiro,¹⁰ Ernesto Carvalho,¹⁰ Pedro Oliveira,¹¹ José M Lopes,^{4,5} Rui M Reis^{1,2,12}

¹Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ²ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal; ³Department of Neurosurgery, Hospital S. João, Porto, Portugal; ⁴IPATIMUP – Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; ⁵Medical Faculty, Department of Pathology, University of Porto, Porto, Portugal; Departments of ⁶Pathology, ⁷Oncology, ⁸Neurosurgery, Hospital S. Marcos, Braga, Portugal; ⁹Unit of Neuropathology, ¹⁰Department of Neurosurgery, Hospital S. Antonio, Porto, Portugal; ¹¹Department of Population Studies, ICBAS, University of Porto, Porto, Portugal; ¹²Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil.

Cancer Epidemiology, Biomarkers & Prevention



Impact of *EGFR* Genetic Variants on Glioma Risk and Patient Outcome

Bruno Marques Costa, Marta Viana-Pereira, Ricardo Fernandes, et al.

Cancer Epidemiol Biomarkers Prev 2011;20:2610-2617. Published OnlineFirst September 29, 2011.

Updated Version

Access the most recent version of this article at:
doi:[10.1158/1055-9965.EPI-11-0340](https://doi.org/10.1158/1055-9965.EPI-11-0340)

Supplementary Material

Access the most recent supplemental material at:
<http://cebp.aacrjournals.org/content/suppl/2011/09/27/1055-9965.EPI-11-0340.DC1.html>

Cited Articles

This article cites 43 articles, 23 of which you can access for free at:
<http://cebp.aacrjournals.org/content/20/12/2610.full.html#ref-list-1>

E-mail alerts

[Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Research Article

Impact of *EGFR* Genetic Variants on Glioma Risk and Patient Outcome

Bruno Marques Costa^{1,2}, Marta Viana-Pereira^{1,2}, Ricardo Fernandes^{1,2}, Sandra Costa^{1,2}, Paulo Linhares³, Rui Vaz³, Céline Pinheiro^{1,2}, Jorge Lima⁴, Paula Soares^{4,5}, Ana Silva⁶, Fernando Pardal⁶, Júlia Amorim⁷, Rui Nabico⁷, Rui Almeida⁸, Carlos Alegria⁸, Manuel Melo Pires⁹, Célia Pinheiro¹⁰, Ernesto Carvalho¹⁰, Pedro Oliveira¹¹, José M. Lopes^{4,5}, and Rui M. Reis^{1,2,12}

Abstract

Background: The epidermal growth factor receptor (EGFR) regulates important cellular processes and is frequently implicated in human tumors. Three *EGFR* polymorphisms have been described as having a transcriptional regulatory function: two single-nucleotide polymorphisms in the essential promoter region, –216G/T and –191C/A, and a polymorphic (CA)_n microsatellite sequence in intron 1. We aimed to elucidate the roles of these *EGFR* polymorphisms in glioma susceptibility and prognosis.

Methods: We conducted a case-control study with 196 patients with glioma and 168 cancer-free controls. Unconditional multivariate logistic regression models were used to calculate ORs and 95% confidence intervals. A Cox regression model was used to evaluate associations with patient survival. False-positive report probabilities were also assessed.

Results: None of the *EGFR* –216G/T variants was significantly associated with glioma risk. The –191C/A genotype was associated with higher risk for glioma when the (CA)_n alleles were classified as short for ≤16 or ≤17 repeats. Independently of the (CA)_n repeat cutoff point used, shorter (CA)_n repeat variants were significantly associated with increased risk for glioma, particularly glioblastoma and oligodendroglioma. In all tested models with different (CA)_n cutoff points, only –191C/A genotype was consistently associated with improved survival of patients with glioblastoma.

Conclusions: Our findings implicate *EGFR* –191C/A and the (CA)_n repeat polymorphisms as risk factors for gliomas, and suggest –191C/A as a prognostic marker in glioblastoma.

Impact: Our data support a role of these *EGFR* polymorphisms in determining glioma susceptibility, with potential relevance for molecularly based stratification of patients with glioblastoma for individualized therapies. *Cancer Epidemiol Biomarkers Prev*; 20(12); 2610–7. ©2011 AACR.

Introduction

Glioma is a broad category of tumors divided into histologic subgroups based on the type of glial cell of origin or morphologic similarities between tumor and normal glial cells: astrocytomas (astrocytic lineage), oligodendrogliomas (oligodendroglial lineage), and oligoastrocytomas (mixed lineage) are the major subtypes, whereas ependymomas (ependymal lineage) are less common (1, 2). They are the most common primary central nervous system tumors and account for approximately 80% of those that are malignant (2), for which efficient therapies are not available (3–5). Despite recent advances in the field of neuro-oncology, the prognosis of patients with glioma remains very poor (6); in particular, patients with glioblastoma, the most common and aggressive (WHO grade IV) form of glioma, present median survival time ranging from 12 to 15 months and the majority die within 2 years (5). Their etiology remains largely unknown: so far, only exposure to high-dose therapeutic

Authors' Affiliations: ¹Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ²ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal; ³Department of Neurosurgery, Hospital S. João, Porto, Portugal; ⁴IPATIMUP - Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; ⁵Medical Faculty, Department of Pathology, University of Porto, Porto, Portugal; ⁶Departments of ⁶Pathology, ⁷Oncology, ⁸Neurosurgery, Hospital S. Marcos, Braga, Portugal; ⁹Unit of Neuropathology, ¹⁰Department of Neurosurgery, Hospital S. António, Porto, Portugal; ¹¹Department of Population Studies, ICBAS, University of Porto, Porto, Portugal; ¹²Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

B.M. Costa and M. Viana-Pereira contributed equally to this work.

Corresponding Author: Rui M. Reis, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, Braga 4710-057, Portugal. Phone: 351253604825; Fax: 351253604820; E-mail: rreis@ecsaude.uminho.pt

doi: 10.1158/1055-9965.EPI-11-0340

©2011 American Association for Cancer Research.

radiation has been firmly established as a risk factor, but other plausible causes include genetic syndromes, familial aggregation, and genetic polymorphisms (7, 8). Equally mysterious are truly clinically relevant prognostic factors for patients with glioma; patient age and clinical performance status are clearly associated with patient outcome, but recent evidences suggest that molecular traits of tumor may also be important determinants of prognosis (9–11).

The epidermal growth factor receptor (EGFR) pathway plays prominent roles in regulating cell growth, apoptosis, and differentiation, a process that is tightly regulated in normal epithelial cells (12). Deregulation of this pathway can occur, for example, via somatic *EGFR* mutations (e.g., *EGFRvIII*), gene amplifications, and ligand-independent constitutive stimulation signaling loops, and has been implicated in several human tumors, including gliomas (13). Aberrant EGFR signaling may ultimately affect major hallmarks of cancer including tumor growth, invasion, malignancy, and prognosis (13–16). In addition to these somatic molecular alterations, germ line polymorphic variants in the EGF/EGFR pathway have also been implicated in tumor risk, therapy response, and patient outcome (17–20). We have previously shown that a functional *EGF* polymorphism (*EGF* +61) is associated with glioma risk (19), which adds to the growing hypothesis that polymorphisms in the EGFR pathway may be relevant in the context of glioma.

Two single-nucleotide polymorphisms (SNP) were recently found in the essential promoter region of the *EGFR* gene (–216G/T and –191C/A), and the T variant in the –216 SNP was shown to increase the promoter activity in an Sp1-dependent manner, resulting in higher gene expression both *in vitro* and *in vivo* (21). A study by Carpentier and colleagues (22) implicated the –216T variant as a risk factor for glioblastoma. Likewise, a highly polymorphic microsatellite sequence (CA)_n repeat was identified in the first intron of *EGFR* (23). It has been proposed that transcriptional activity of *EGFR* declines with increasing number of

(CA)_n repeats, suggesting that this polymorphism may play a role in cancer susceptibility (24).

In the context of the widely acknowledged role of EGFR signaling in gliomas and the functional relevance of particular *EGFR* polymorphisms, we investigated the relevance of *EGFR* –216G/T, –191C/A, and (CA)_n repeat polymorphisms in glioma susceptibility and patient prognosis. To do so, we conducted a population-based case-control study of 196 patients with glioma and 168 cancer-free controls from Portugal.

Materials and Methods

Study population

We studied a total of 196 patients with glioma recruited from Hospital S. Marcos, Braga, Hospital S. João, and Hospital S. António. Tumors were classified by experienced neuropathologists according to WHO standards (1). The control group is composed of 168 cancer-free individuals randomly selected from blood donors' bank. All patients and controls were from Northwest Portugal and of Caucasian ethnicity. Table 1 summarizes the clinicopathologic features of patients and controls. This study was conducted in accordance with institutional ethical committees. Blood samples were obtained following informed signed consent. All the samples enrolled were unlinked and unidentified from their donors.

Genotyping

Genomic DNA from patients with glioma was obtained from blood (*n* = 70) or formalin-fixed, paraffin-embedded tumor samples (*n* = 126) as previously reported (19). *EGFR* –216G/T and –191C/A genotyping was carried out by PCR amplification of the promoter region from nucleotides –352 to –155 (Fw primer: 5'-CTCCTCC-TCTCTGCTCTC-3'; Rv primer: 5'-GGGGCTAGC-TCGGGACTC-3'), followed by RFLP with *Bse*RI and *Sac*II respectively, as previously described (21). To evaluate

Table 1. Clinicopathologic features of gliomas and controls

Groups (WHO grade)	Number of cases	Age, y (mean ± SD)	Male/female ratio
Controls	168	44.2 ± 15.6	1.43
Gliomas (II–IV)	196	52.0 ± 14.1	1.23
Astrocytoma (II–IV)	136	54.3 ± 13.5	1.19
Diffuse astrocytoma (II)	15	40.1 ± 13.5	0.36
Anaplastic astrocytoma (III)	5	37.4 ± 11.6	All males
Glioblastoma (IV)	113	56.8 ± 12.1	1.31
Gliosarcoma (IV)	3	59.7 ± 5.69	0.50
Oligodendroglioma (II–III)	58	47.0 ± 14.3	1.32
Oligodendroglioma (II)	30	42.2 ± 16.1	1.31
Anaplastic oligodendroglioma (III)	28	52.0 ± 10.0	1.33
Oligoastrocytoma	2	42.0 ± 8.49	1.00
Anaplastic oligoastrocytoma (III)	2	42.0 ± 8.49	1.00

EGFR (CA)_n repeats in intron 1, a 5-carboxyfluorescein (5-FAM)-labeled PCR reaction was carried out (Fw primer: 5'-FAM-labeled 5'-GGCTCACAGCAAACCTCTCC-3'; Rv primer: 5'-AAGCCAGACTCGCTCATGTT-3') and the products subsequently analyzed by single capillary genetic analysis, as previously described (25). Briefly, PCR cycles were 95°C for 7 minutes; followed by 35 cycles of 95°C for 45 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and a final step at 72°C for 7 minutes. PCR fragments ranging from 194 to 212 bp containing the polymorphic region were obtained. One microliter of PCR product and 0.6 µL of GeneScan 500 TAMRA size standard (Applied Biosystems) were mixed with 14.4 µL of formamide. PCR products were then separated using an ABI Prism 310 (Applied Biosystems), and the fragment lengths were determined with GeneScan Analysis software version 3.7 (Applied Biosystems).

Data analyses and statistical methods

EGFR -216G/T and -191C/A groups were categorized as previously described (21). Because *EGFR* (CA)_n repeats vary between $n = 14$ and $n = 23$ and considering different approaches used in other studies (26–30), we evaluated *EGFR* (CA)_n repeat length on the basis of 2 different methods. First, by the genotype-based method (26, 30), the genotypes of *EGFR* (CA)_n repeat were categorized into 3 groups considering a cutoff point of 17 (CA) repeats: homozygous short (SS) contained 2 short alleles (both alleles ≤ 17 CA repeats), homozygous long (LL) contained 2 long alleles (both alleles > 17 CA repeats), and heterozygous short/long (SL) contained one short (≤ 17) and one long (> 17) allele. Because the cutoff value to consider short and long alleles has been somewhat controversial in the literature (30–33), we also tested 2 additional cutoff values to define short alleles with ≤ 16 and ≤ 18 CA repeats, and long alleles with > 16 and > 18 CA repeats, respectively (33). Second, we investigated the *EGFR* (CA)_n repeat polymorphism by the summed allele lengths method (28), where individuals were categorized into short (sum of alleles length < 36) or long (sum of alleles length ≥ 36).

The χ^2 test was used to assess whether the observed allele distributions of all polymorphisms were in Hardy-Weinberg equilibrium in the control group. ORs and 95% confidence intervals (95% CI) for the effect of *EGFR* variants on the risk for each glioma type were estimated by multivariate logistic regression analyses, adjusted for *EGF* +61A/G genotype, which was previously reported to affect glioma susceptibility (19), and patient age (as a continuous variable) and sex. The false-positive report probability (FPRP) was calculated for significant associations observed in multivariate tests according to the study of Wacholder and colleagues (34). Associations between *EGFR* variants [-216G/T, -191C/A, and (CA)_n repeat] and patient survival were assessed using a multivariate Cox regression model adjusted for *EGF* +61A/G, patient age, and sex (35).

Results

EGFR -216G/T, -191C/A, and (CA)_n repeat polymorphisms and risk of glioma

We studied a total of 196 patients with glioma and 168 cancer-free control individuals. Successful determination of *EGFR* genotypes was achieved for all samples. The distributions of *EGF* +61A/G, *EGFR* -216G/T, and (CA)_n allele frequencies in the control group were in Hardy-Weinberg equilibrium ($P = 0.121$, $P = 0.899$, and $P = 0.150$, respectively); however, *EGFR* -191C/A alleles in controls were not in equilibrium ($P = 0.034$). A summary of clinicopathologic features of the controls and cases is presented in Table 1.

The frequencies of each *EGFR* genotype in controls and cases are presented in Table 2. The *EGFR* allele frequencies in our cancer-free control population are similar to those found in other control populations (21, 31, 33). The most frequent genotypes for each polymorphism in the control group (homozygous -216G/G, homozygous -191C/C, and heterozygous (CA)_n repeat SL) were considered as references. To assess associations between each variant of the studied *EGFR* polymorphisms and risk for glioma, we used an unconditional multivariate logistic regression analysis adjusted for potential confounding variables (*EGF* +61A/G genotype, patient age, and sex). The control group was compared with all glioma cases (WHO grades II–IV) and also with glioblastoma (WHO grade IV) and oligodendroglioma (WHO grades II and III; Table 2), as these were the most frequent subtypes in our series (Table 1).

No statistically significant associations were found between *EGFR* -216G/T genotype variants and risk for glioma, glioblastoma, or oligodendroglioma (Table 2; all values of $P > 0.05$). In contrast, when compared with the -191C/C reference genotype, the heterozygous -191C/A genotype was significantly associated with increased risk for glioma (OR = 1.81; 95% CI, 1.01–3.24; Table 2). No significant associations between the -191A/A genotype and risk for glioma, glioblastoma, or oligodendroglioma were found (Table 2; all values of $P > 0.05$). In addition, the SS genotype of (CA)_n repeat (considering short alleles ≤ 17 CA repeats) was significantly associated with increased risk for glioma (OR = 2.38; 95% CI, 1.42–3.98), glioblastoma (OR = 2.25; 95% CI, 1.20–4.25), and oligodendroglioma (OR = 2.45; 95% CI, 1.17–5.12), as well as the LL genotype was significantly associated with increased risk for glioma (OR = 1.95; 95% CI, 1.02–3.73). As expected (19), *EGF* +61A/G and G/G genotypes were significantly associated with increased risk for glioma (OR = 1.75; 95% CI, 1.03–2.96 and OR = 1.90; 95% CI, 1.03–3.52, respectively; Table 2), particularly oligodendroglioma (OR = 2.80; 95% CI, 1.18–6.67 and OR = 2.75; 95% CI, 1.06–7.09, respectively; Table 2) and nearly statistically significant for glioblastoma (OR = 1.72; 95% CI, 0.91–3.26 and OR = 1.92; 95% CI, 0.91–4.03, respectively; Table 2).

When other cutoff values were used to classify short and long alleles for the (CA)_n repeat polymorphism,

Table 2. Multivariate logistic regression analysis of associations between *EGFR/EGF* polymorphisms and risk for glioma groups

Polymorphism	Control	Glioma (grades II–IV)	OR (95% CI)	Glioblastoma (grade IV)	OR (95% CI)	Oligodendroglioma (grades II–III)	OR (95% CI)
<i>EGFR</i> –216G/T							
G/G	77	91	—	55	—	28	—
G/T	74	85	1.12 (0.68–1.84)	45	1.09 (0.58–2.03)	25	1.01 (0.49–2.07)
T/T	17	20	1.15 (0.52–2.59)	13	1.30 (0.50–3.40)	5	1.02 (0.31–3.40)
<i>EGFR</i> –191C/A							
C/C	130	140	—	77	—	41	—
C/A	32	49	1.81 (1.01–3.24)	30	1.97 (0.96–4.03)	16	2.08 (0.93–4.63)
A/A	6	7	0.68 (0.19–2.45)	6	0.88 (0.21–3.70)	1	0.47 (0.05–4.84)
<i>EGFR</i> (CA) _n repeat ^a							
SL	92	75	—	42	—	23	—
LL	28	40	1.95 (1.02–3.73)	25	2.14 (0.96–4.76)	10	1.83 (0.68–4.94)
SS	48	81	2.38 (1.42–3.98)	46	2.25 (1.20–4.25)	25	2.45 (1.17–5.12)
<i>EGF</i> +61A/G							
A/A	57	47	—	30	—	9	—
A/G	73	96	1.75 (1.03–2.96)	54	1.72 (0.91–3.26)	32	2.80 (1.18–6.67)
G/G	38	53	1.90 (1.03–3.51)	29	1.92 (0.91–4.03)	17	2.75 (1.06–7.09)
Age			1.04 (1.02–1.06)		1.07 (1.04–1.09)		1.02 (1.00–1.04)
Sex							
Female	69	88	—	49	—	25	—
Male	99	108	0.95 (0.60–1.49)	64	1.02 (0.59–1.78)	33	0.98 (0.51–1.88)

NOTE: Bold-faced values indicate significant difference at 5% level.

^a(CA)_n repeat considered short ≤ 17 and long > 17.

specifically $n = 16$ or $n = 18$ (i.e., $S \leq 16$ and $L > 16$, or $S \leq 18$ and $L > 18$, respectively), similar results were obtained; particularly, the (CA) repeat homozygous SS genotype was again significantly associated with increased risks for glioma, glioblastoma, and oligodendroglioma both for a cutoff value of $S \leq 16$ repeats (Supplementary Table S1) and $S \leq 18$ repeats (Supplementary Table S2). The LL genotype was also associated with increased risk for glioma and glioblastoma for the cutoff value of $S \leq 16$ (Supplementary Table S1) and for glioblastoma only for the cutoff value of $S \leq 18$ (Supplementary Table S2). Of note, the heterozygous –191C/A genotype was also associated with increased risk for glioma when the cutoff value for (CA)_n short and long repeats was 16 (Supplementary Table S1) but not in the case of a cutoff value of 18 repeats (Supplementary Table S2). We also analyzed the associations between *EGFR* variants and risk considering the sum of alleles for the (CA)_n repeat polymorphism (28), which in our series varied from 29 to 41 repeats. Because the median value for the sum of alleles in the control group was 36, we categorized the sum of alleles as short <36 and long ≥ 36 but none of the *EGFR* variants were significantly associated with risk in this analysis (Supplementary Table S3).

The calculation of FPRP showed that all of the above-mentioned *EGFR* associations with risk remained noteworthy (FPRP ≤ 0.5) when a prior probability of associ-

ation of 10% or greater was considered (Table 3). This was also the case for *EGF* +61A/G associations, except in the case of the G/G genotype in oligodendroglioma risk (FPRP = 0.562; Table 3). For a prior probability of 5% or more, only the associations between (CA)_n SS genotype and risk for glioma and glioblastoma remained noteworthy, which remained significant even for a prior probability of 1% in the case of glioma risk (Table 3).

Taken together, these data strongly suggest that shorter variants of the *EGFR* intron 1 (CA)_n repeat polymorphism increase the risk for gliomas, particularly glioblastoma and oligodendroglioma.

***EGFR* –216G/T, –191C/A, and (CA)_n repeat polymorphisms and survival of patients with glioblastoma**

In a subset of patients with glioblastoma, we also had available follow-up data ($n = 63$). Thus, we investigated the associations between each *EGFR* variant (–216G/T, –191C/A, and (CA)_n repeat) and overall survival by a multivariate Cox proportional hazard model, adjusted for *EGF* +61A/G, patient age, and sex.

None of the *EGFR* –216G/T variants was associated with survival of patients with glioblastoma (Table 4; Supplementary Tables S4–S6 when the (CA)_n repeat variants were classified considering a cutoff value of $S \leq 17$, $S \leq 16$, $S \leq 18$, or sum of alleles < 36, respectively).

Costa et al.

Table 3. FPRP for significant associations with risk

Polymorphism	OR (95% CI)	Power ^a	Reported <i>P</i>	Prior probability			
				0.1	0.05	0.01	0.001
Glioma risk							
<i>EGFR</i> -191C/A (C/C ref.)	1.81 (1.01–3.24)	0.632	0.046	0.395	0.579	0.878	0.980
<i>EGFR</i> (CA) _n LL (SL ref.)	1.95 (1.02–3.73)	0.530	0.044	0.425	0.609	0.890	0.988
<i>EGFR</i> (CA) _n SS (SL ref.)	2.38 (1.42–3.98)	0.254	0.001	0.033	0.066	0.270	0.789
<i>EGF</i> +61A/G (A/A ref.)	1.75 (1.03–2.96)	0.691	0.037	0.325	0.504	0.841	0.982
<i>EGF</i> +61G/G (A/A ref.)	1.90 (1.03–3.51)	0.565	0.040	0.391	0.576	0.876	0.986
Glioblastoma risk							
<i>EGFR</i> (CA) _n SS (SL ref.)	2.25 (1.20–4.25)	0.358	0.012	0.238	0.398	0.775	0.972
Oligodendroglioma risk							
<i>EGFR</i> (CA) _n SS (SL ref.)	2.45 (1.17–5.12)	0.295	0.017	0.344	0.525	0.852	0.998
<i>EGF</i> +61A/G (A/A ref.)	2.80 (1.18–6.67)	0.224	0.020	0.447	0.630	0.899	0.989
<i>EGF</i> +61G/G (A/A ref.)	2.75 (1.06–7.09)	0.255	0.036	0.562	0.730	0.934	0.993

NOTE: Bold-faced values indicate the FPRP ≤ 0.5 for the most likely prior probability.^aEstimation of statistical power to detect an OR of 2.0 with an α level equal to the observed *P* value.

In contrast, the heterozygous -191C/A genotype was significantly associated with improved overall survival of patients with glioblastoma, as compared with homozygous -191C/C, which was consistent across all analyses, that is, when (CA)_n alleles were classified as $S \leq 17$ (OR = 0.37; 95% CI, 0.16–0.88; Table 4), $S \leq 16$ (OR = 0.35; 95% CI, 0.15–0.82; Supplementary Table S4), $S \leq 18$ (OR = 0.37; 95% CI, 0.16–0.82; Supplementary Table S5), or sum of alleles < 36 (OR = 0.39; 95% CI, 0.17–0.86; Supplementary Table S6).

The homozygous SS and LL (CA)_n repeat genotypes were also significantly associated with a longer survival of patients with glioblastoma when the (CA)_n repeat variants were classified as $S \leq 17$ (OR = 0.33; 95% CI, 0.11–0.95 for LL genotype and OR = 0.41; 95% CI, 0.18–0.93 for SS genotype; Table 4). In the case of the SS genotype, this association with longer survival was maintained when a cutoff value of $S \leq 16$ was considered (OR = 0.42; 95% CI, 0.18–0.95; Supplementary Table S4).

FPRP calculations showed that the association between -191C/A and (CA)_n repeat SS genotypes and longer survival of patients with glioblastoma remained noteworthy for a prior probability of association of 10% or more (FPRP < 0.5 for all associations), which was not the case for LL genotypes (FPRP > 0.5 , data not shown).

Taken together, these data strongly suggest that *EGFR* -191C/A and intron 1 (CA)_n repeat polymorphisms are prognostic markers in patients with glioblastoma, whereas -216G/T variants do not seem to predict the outcome of patients with glioblastoma.

Discussion

Gliomas are the most frequent and malignant primary central nervous system tumors. These result in more years

of life lost than do any other tumors (36) and are a significant source of cancer-related death (37). It is generally assumed that genetic and environmental factors contribute to gliomagenesis, but the etiology of gliomas remains very poorly understood. Presently, one of the several lines of brain tumor research focuses on the relevance of germ line genetic polymorphisms in glioma risk, grade, prognosis, and response to specific therapies. We have recently shown that an SNP in the *EGF* gene (which encodes one of the main *EGFR* ligands), *EGF* +61A/G, has functional consequences and associated the G allele with increased risk for glioma, particularly glioblastoma and oligodendroglioma (19).

The *EGFR* pathway is commonly altered in gliomas. Approximately 50% of glioblastomas show *EGFR* amplification and overexpression, 40% of which express the mutant form *EGFRvIII*, resulting in constitutive activation of the *EGFR* pathway (13, 38–40). Previously, Carpentier and colleagues (22) showed an association between the -216T allele and increased risk for glioblastoma. We attempted to replicate these findings, and examined, for the first time, the implication of -216G/T and -191C/A SNPs in other types of glioma. In opposition to the results of the study of Carpentier and colleagues, we have not seen association between *EGFR* -216T allele and increased risk for glioblastomas or any other glioma subtype. This discrepancy may be partially explained by distinct population sampling and the fact that we conducted a logistic regression adjusted for 2 other *EGFR* polymorphisms, together with *EGF* +61A/G, patient age, and sex. Importantly, the allele distribution of the -216G/T polymorphism in the control cancer-free population, we report here (67.9% G, 32.1% T, $n = 168$) is very similar to that reported by other studies [68.3% G, 31.7% T, $n = 60$ (ref. 21); 68.2% G, 31.8% T, $n = 22$ (ref. 41)]. Inversely, the study of

Table 4. Multivariate Cox proportional hazard model analysis of associations between *EGFR*/*EGF* polymorphisms and survival of patients with glioblastoma (adjusted for patient age and sex)

Polymorphism	Glioblastoma (grade IV)	OR (95% CI)
<i>EGFR</i> -216G/T		
G/G	33	—
G/T	23	0.49 (0.23–1.01)
T/T	7	0.61 (0.21–1.73)
<i>EGFR</i> -191C/A		
C/C	41	—
C/A	18	0.37 (0.16–0.88)
A/A	4	0.56 (0.12–2.69)
<i>EGFR</i> (CA) _n repeat ^a		
SL	17	—
LL	17	0.33 (0.11–0.95)
SS	29	0.41 (0.18–0.93)
<i>EGF</i> +61A/G		
A/A	16	—
A/G	28	1.06 (0.47–2.38)
G/G	19	2.10 (0.89–4.99)
Age		1.00 (0.97–1.03)
Sex		
Female	25	—
Male	38	1.02 (0.54–1.92)

NOTE: Bold-faced values indicate significant difference at 5% level.

^a(CA)_n repeat considered short ≤ 17 and long > 17.

Carpentier and colleagues shows a significantly different distribution of -216G/T alleles in the control group (53.2% G, 46.8% T, $n = 176$) when compared with our and others data [$\chi^2(3) = 19.4, P < 0.001$] (21, 41). Even though the allele frequencies of this SNP vary greatly based on the ethnic background (21), this feature cannot explain the observed differences because all patients in our and others studies (21, 22, 41) were Caucasians. Concerning the -191C/A SNP, we observed that the heterozygous -191C/A genotype was associated with increased risk for glioma. A recent study by Schwartzbaum and colleagues (42) identified 3 *EGFR* SNPs consistently associated with glioblastoma risk across 4 independent data sets. In addition, these SNPs were highly correlated with 4 other *EGFR* SNPs previously found to be significantly associated with risk for glioma (43). Collectively, these and our data seem to support *EGFR* SNPs as potential risk factors for glioma.

Several studies suggest that *EGFR* expression is dependent on the number of the intron 1 (CA)_n repeats (28, 30, 44, 45). In addition, this polymorphism has been associated with risk of breast, lung, and colorectal cancers

(31–33) but was never studied in gliomas. In this study, we provide the first evidence on the relevance of the *EGFR* (CA)_n repeat length polymorphism in glioma risk and patient survival. Because different criteria have been published for the analysis of this polymorphism and no consensual cutoff point exists to distinguish short and long *EGFR* (CA)_n repeat alleles (30–33), we used 3 different cutoff points to cover most of the previously published analysis (considering short alleles ≤16, ≤17, or ≤18 CA repeats) and evaluated the (CA)_n repeat by the genotype, and the sum of the alleles length (considering the cutoff point for the sum of alleles as the median value in our control group). Our data show that the homozygous SS genotype of the (CA)_n repeat polymorphism was associated with increased risk for glioma, glioblastoma, or oligodendroglioma, regardless of the selected cutoff point. The homozygous LL genotype was also found to be associated with increased risk for glioma and glioblastoma but only in some of the tested cutoff values. Thus, caution must be taken in the interpretation of the results and validation in an independent series is required.

Investigating the prognostic value of these 3 *EGFR* polymorphisms, we found a significant association between the heterozygous -191C/A genotype and improved survival of patients with glioblastoma, regardless of the criteria used for the cutoff value of the (CA)_n repeat genotypes in the multivariate Cox model (Table 4, Supplementary Tables S4–S6). The SS and LL genotypes for the (CA)_n repeat polymorphism were also associated with a better survival of patients with glioblastoma but only for specific cutoff values (SS when $S \leq 17$ or $S \leq 16$; LL when $S \leq 17$); thus, the clinical relevance of this polymorphism warrants further confirmation.

In our study, the genotype assays of most patients with glioma were carried out in tumor tissue, raising the possibility that somatic alterations in the *EGFR* locus (7p12) could lead to misgenotyping. However, we believe that this is not the case because of the following reasons: (i) the overall distribution of *EGFR* -216 and -191 and (CA)_n repeat genotypes and alleles was not statistically different for the group of patients whose genotyping was done in DNA from blood or tumor tissue ($P = 0.241$ for *EGFR* -216; $P = 0.176$ for *EGFR* -191; $P = 0.155$ for (CA)_n repeats; data not shown); (ii) genotyping of all 3 polymorphisms was done with 100% concordance in 20 glioma cases from whom DNA was available from both peripheral blood and tumor tissue; (iii) we found no statistically significant associations between *EGFR* polymorphic variants and particular *EGFR* molecular alterations (*EGFR* amplification and *EGFRvIII* mutation; Supplementary Table S7) we had previously analyzed in a subset of tumors (13). Accordingly, in lung cancer, it was previously shown for the (CA)_n repeats polymorphism that regardless the amplification status there was 100% concordance in the genotyping of tumor and nontumor tissues of 450 cases (15).

In summary, our data consistently indicate that *EGFR* intron 1 homozygous (CA)_n repeat short genotypes confer higher susceptibility to develop different histologic entities of glioma, and implicate the heterozygous -191C/A genotype as a predictive marker of worse survival in patients with glioblastoma. Because *EGFR* is one of the most frequently altered molecules in high-grade glioma, it is natural to think of it as an attractive therapeutic target. Therefore, further studies are warranted to investigate how these *EGFR* polymorphisms may affect response of patients with glioma to *EGFR*-targeting therapies.

Disclosure of Potential Conflicts of interest

R.M. Reis has commercial research grant from Schering-Plough, Portugal. No potential conflicts of interest were disclosed by other authors.

Acknowledgments

The authors thank the Immunochemotherapy Department of Hospital S. Marcos, and Clínica Laboratorial Dr. Edgar Botelho Moniz, S. Tirso, Portugal, for their helpful assistance in the management of controls.

Grant Support

The study was supported by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/33612/2009; SFRH/BD/29145/2006). Schering-Plough Farma, Portugal.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 11, 2011; revised August 17, 2011; accepted September 21, 2011; published OnlineFirst September 28, 2011.

References

- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. WHO classification of tumours of the central nervous system. Lyon, France: IARC; 2007.
- CBTRUS. Statistical report: primary brain tumors in the United States, 1998–2002. Hinsdale (IL): CBTRUS; 2005.
- Reardon DA, Rich JN, Friedman HS, Bigner DD. Recent advances in the treatment of malignant astrocytoma. *J Clin Oncol* 2006;24:1253–65.
- Jaekle KA, Ballman KV, Rao RD, Jenkins RB, Buckner JC. Current strategies in treatment of oligodendroglioma: evolution of molecular signatures of response. *J Clin Oncol* 2006;24:1246–52.
- Clarke J, Butowski N, Chang S. Recent advances in therapy for glioblastoma. *Arch Neurol* 2010;67:279–83.
- Butowski NA, Sneed PK, Chang SM. Diagnosis and treatment of recurrent high-grade astrocytoma. *J Clin Oncol* 2006;24:1273–80.
- Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. *Acta Neuropathol (Berl)* 2005;109:93–108.
- Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, et al. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. *Cancer* 2008;113:1953–68.
- Costa BM, Smith JS, Chen Y, Chen J, Phillips HS, Aldape KD, et al. Reversing HoxA9 oncogene activation by PI3K inhibition: epigenetic mechanism and prognostic significance in human glioblastoma. *Cancer Res* 2010;70:453–62.
- Hegi ME, Diserens AC, Gorlia T, Hamou MF, de TN, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352:997–1003.
- Phillips HS, Kharbada S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006;9:157–73.
- Yarden Y, Slivkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Viana-Pereira M, Lopes JM, Little S, Milanezi F, Basto D, Pardal F, et al. Analysis of *EGFR* overexpression, *EGFR* gene amplification and the *EGFR* mutation in Portuguese high-grade gliomas. *Anticancer Res* 2008;28:913–20.
- Zhu Y, Parada LF. The molecular and genetic basis of neurological tumours. *Nat Rev Cancer* 2002;2:616–26.
- Nomura M, Shigematsu H, Li L, Suzuki M, Takahashi T, Estess P, et al. Polymorphisms, mutations, and amplification of the *EGFR* gene in non-small cell lung cancers. *PLoS Med* 2007;4:e125.
- Weiss WA, Burns MJ, Hackett C, Aldape K, Hill JR, Kuriyama H, et al. Genetic determinants of malignancy in a mouse model for oligodendroglioma. *Cancer Res* 2003;63:1589–95.
- Araujo A, Ribeiro R, Azevedo I, Coelho A, Soares M, Sousa B, et al. Genetic polymorphisms of the epidermal growth factor and related receptor in non-small cell lung cancer—a review of the literature. *Oncologist* 2007;12:201–10.
- Zhang YM, Cao C, Liang K. Genetic polymorphism of epidermal growth factor 61A>G and cancer risk: a meta-analysis. *Cancer Epidemiol* 2010;34:150–6.
- Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A, et al. Association between functional *EGF*+61 polymorphism and glioma risk. *Clin Cancer Res* 2007;13:2621–6.
- Liu W, He L, Ramirez J, Krishnaswamy S, Kanteti R, Wang YC, et al. Functional *EGFR* germline polymorphisms may confer risk for *EGFR* somatic mutations in non-small cell lung cancer, with a predominant effect on exon 19 microdeletions. *Cancer Res* 2011;71:2423–7.
- Liu W, Innocenti F, Wu MH, Desai AA, Dolan ME, Cook EH Jr, et al. A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter. *Cancer Res* 2005;65:46–53.
- Carpentier C, Laigle-Donadey F, Marie Y, Auger N, Benouaich-Amiel A, Lejeune J, et al. Polymorphism in Sp1 recognition site of the *EGF* receptor gene promoter and risk of glioblastoma. *Neurology* 2006;67:872–4.
- Chi DD, Hing AV, Helms C, Steinbrueck T, Mishra SK, Donis-Keller H. Two chromosome 7 dinucleotide repeat polymorphisms at gene loci epidermal growth factor receptor (*EGFR*) and pro alpha 2 (I) collagen (*COL1A2*). *Hum Mol Genet* 1992;1:135.
- Gebhardt F, Zanker KS, Brandt B. Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. *J Biol Chem* 1999;274:13176–80.
- Butler JM, Buel E, Crivellente F, McCord BR. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. *Electrophoresis* 2004;25:1397–412.
- Kang D, Gridley G, Huang WY, Engel LS, Winn DM, Brown LM, et al. Microsatellite polymorphisms in the epidermal growth factor receptor (*EGFR*) gene and the transforming growth factor-alpha (*TGFA*) gene and risk of oral cancer in Puerto Rico. *Pharmacogenet Genomics* 2005;15:343–7.
- McKay JA, Murray LJ, Curran S, Ross VG, Clark C, Murray GI, et al. Evaluation of the epidermal growth factor receptor (*EGFR*) in colorectal tumours and lymph node metastases. *Eur J Cancer* 2002;38:2258–64.
- Amador ML, Oppenheimer D, Perea S, Maitra A, Cusatis G, Iacobuzio-Donahue C, et al. An epidermal growth factor receptor intron 1 polymorphism mediates response to epidermal growth factor receptor inhibitors. *Cancer Res* 2004;64:9139–43.
- Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci U S A* 1997;94:3320–3.

30. Etienne-Grimaldi MC, Pereira S, Magne N, Formento JL, Francoual M, Fontana X, et al. Analysis of the dinucleotide repeat polymorphism in the epidermal growth factor receptor (EGFR) gene in head and neck cancer patients. *Ann Oncol* 2005;16:934-41.
31. Zhang W, Weissfeld JL, Romkes M, Land SR, Grandis JR, Siegfried JM. Association of the EGFR intron 1 CA repeat length with lung cancer risk. *Mol Carcinog* 2007;46:372-80.
32. Zhang W, Park DJ, Lu B, Yang DY, Gordon M, Groshen S, et al. Epidermal growth factor receptor gene polymorphisms predict pelvic recurrence in patients with rectal cancer treated with chemoradiation. *Clin Cancer Res* 2005;11:600-5.
33. Brandt B, Hermann S, Straif K, Tidow N, Buerger H, Chang-Claude J. Modification of breast cancer risk in young women by a polymorphic sequence in the egfr gene. *Cancer Res* 2004;64:7-12.
34. Wacholder S, Chanock S, Garcia-Closas M, El GL, Rothman N. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 2004;96:434-42.
35. Costa BM, Caeiro C, Guimaraes I, Martinho O, Jaraquemada T, Augusto I, et al. Prognostic value of MGMT promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: a Portuguese multicentre study. *Oncol Rep* 2010;23:1655-62.
36. Burnet NG, Jefferies SJ, Benson RJ, Hunt DP, Treasure FP. Years of life lost (YLL) from cancer is an important measure of population burden—and should be considered when allocating research funds. *Br J Cancer* 2005;92:241-5.
37. Jemal A, Siegel R, Ward E, Hao YP, Xu JQ, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225-49.
38. Humphrey PA, Wong AJ, Vogelstein B, Zalutsky MR, Fuller GN, Archer GE, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc Natl Acad Sci U S A* 1990;87:4207-11.
39. Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS, et al. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 1992;89:2965-9.
40. Heimberger AB, Suki D, Yang D, Shi W, Aldape K. The natural history of EGFR and EGFRvIII in glioblastoma patients. *J Transl Med* 2005;3:38.
41. Liu W, Wu X, Zhang W, Montenegro RC, Fackenthal DL, Spitz JA, et al. Relationship of EGFR mutations, expression, amplification, and polymorphisms to epidermal growth factor receptor inhibitors in the NCI60 cell lines. *Clin Cancer Res* 2007;13:6788-95.
42. Schwartzbaum JA, Xiao Y, Liu Y, Tsavachidis S, Berger MS, Bondy ML, et al. Inherited variation in immune genes and pathways and glioblastoma risk. *Carcinogenesis* 2010;31:1770-7.
43. Andersson U, Schwartzbaum J, Wiklund F, Sjöström S, Liu Y, Tsavachidis S, et al. A comprehensive study of the association between the EGFR and ERBB2 genes and glioma risk. *Acta Oncol* 2010;49:767-75.
44. Buerger H, Packeisen J, Boecker A, Tidow N, Kersting C, Bielawski K, et al. Allelic length of a CA dinucleotide repeat in the egfr gene correlates with the frequency of amplifications of this sequence—first results of an inter-ethnic breast cancer study. *J Pathol* 2004;203:545-50.
45. Buerger H, Gebhardt F, Schmidt H, Beckmann A, Huttmacher K, Simon R, et al. Length and loss of heterozygosity of an intron 1 polymorphic sequence of egfr is related to cytogenetic alterations and epithelial growth factor receptor expression. *Cancer Res* 2000;60:854-7.

3 IMMUNOGLOBULIN GENES IMPLICATED IN GLIOMA RISK

Janardan P Pandey,^{1,*} Navtej Kaur,¹ Sandra Costa,^{2,3} Julia Amorim,⁴ Rui Nabico,⁴ Paulo Linhares,^{5,6} Rui Vaz,^{5,6} Marta Viana-Pereira^{2,3} and Rui M Reis^{2,3,7}

¹Department of Microbiology and Immunology; Medical University of South Carolina; Charleston, SC USA; ²Life and Health Sciences Research Institute; University of Minho; Campus de Gualtar; Braga, Portugal; ³IC VS/3B's-PT Government Associate Laboratory; Campus de Gualtar, Braga, Portugal; ⁴Department of Oncology; Hospital de Braga; Sete Fontes - São Victor; Braga, Portugal; ⁵Department of Neurosurgery; Hospital S. João; Porto, Portugal; ⁶Faculty of Medicine; Oporto University; Porto, Portugal; ⁷Molecular Oncology Research Center; Barretos Cancer Hospital; Barretos, SP Brazil.

Immunoglobulin genes implicated in glioma risk

Janardan P Pandey^{1,*}, Navtej Kaur¹, Sandra Costa^{2,3}, Julia Amorim⁴, Rui Nabico⁴, Paulo Linhares^{5,6}, Rui Vaz^{5,6}, Marta Viana-Pereira^{2,3}, and Rui M Reis^{2,3,7}

¹Department of Microbiology and Immunology; Medical University of South Carolina; Charleston, SC USA; ²Life and Health Sciences Research Institute; University of Minho; Campus de Gualtar; Braga, Portugal; ³ICVS/3B's-PT Government Associate Laboratory; Campus de Gualtar, Braga, Portugal; ⁴Department of Oncology; Hospital de Braga; Sete Fontes-São Victor; Braga, Portugal; ⁵Department of Neurosurgery; Hospital S. João; Porto, Portugal; ⁶Faculty of Medicine; Oporto University; Porto, Portugal; ⁷Molecular Oncology Research Center; Barretos Cancer Hospital; Barretos, SP Brazil

Keywords: Glioma, astrocytoma, glioblastoma, GM allotypes, *IGHG* genes, HCMV

Abbreviations: CI, confidence interval; EGFR, epidermal growth factor receptor; FcγR, Fcγ receptor; GM, γ marker; GWAS, genome-wide association studies; HCMV, human cytomegalovirus; HER1, human epidermal growth factor receptor 1; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; HR, hazard ratio; IGHG, immunoglobulin heavy chain γ; *IRL*, internal repeat long; OR, odds ratio; *TRL*, terminal repeat long

Both genetic and environmental factors are thought to be causal in gliomagenesis. Several genes have been implicated in glioma development, but the putative role of a major immunity-related gene complex member, immunoglobulin heavy chain γ (*IGHG*) has not been evaluated. Prior observations that *IGHG*-encoded γ marker (GM) allotypes exhibit differential sensitivity to an immunoevasion strategy of cytomegalovirus, a pathogen implicated as a promoter of gliomagenesis, has lead us to hypothesize that these determinants are risk factors for glioma. To test this hypothesis, we genotyped the *IGHG* locus comprising the GM alleles, specifically GM alleles 3 and 17, of 120 glioma patients and 133 controls via TaqMan® genotyping assay. To assess the associations between GM genotypes and the risk of glioma, we applied an unconditional multivariate logistic regression analysis adjusted for potential confounding variables. In comparison to subjects who were homozygous for the GM 17 allele, the GM 3 homozygotes were over twice as likely, and the GM 3/17 heterozygotes were over three times as likely, to develop glioma. Similar results were achieved when analyzed by combining the data corresponding to alleles GM 3 and GM 3/17 in a dominant model. The GM 3/17 genotype and the combination of GM 3 and GM 3/17 were found to be further associated with over 3 times increased risk for high-grade astrocytoma (grades III-IV). Allele frequency analyses also showed an increased risk for gliomas and high-grade astrocytoma in association with GM 3. Our findings support the premise that the GM 3 allele may present risk for the development of glioma, possibly by modulating immunity to cytomegalovirus.

Introduction

Malignant glioma, the most common primary brain cancer, is divisible into histologic subgroups based on the specific types of glial cells involved. Despite recent advances in therapy, the prognosis for the majority of glioma patients remains grim. The most aggressive form of the disease *glioblastoma multiforme* is commonly lethal, with afflicted patients typically dying approximately 2 y, or less, following diagnosis. Therefore, there is an urgent need of novel routes of investigation in glioma research that might lead to more efficacious therapies to treat this malignancy.

Like other complex diseases, glioma is thought to result from both genetic and environmental factors. Several genes, including some belonging to the immune system, have been implicated in glioma pathogenesis.¹⁻¹⁰ But the putative role of γ marker (GM) allotypes, encoded by three highly polymorphic

IGHG loci¹¹ on chromosome 14, has not been evaluated. There is a strong immunogenetic rationale for investigating the role of GM allotypes in the etiopathogenesis of glioma considering that these determinants modulate an immunoevasion strategy of human cytomegalovirus (HCMV), a common herpes virus that is thought to be an active promoter or oncological modulator of gliomagenesis.¹²⁻¹⁶ In a previous study, we showed that an HCMV-encoded Fcγ receptor (FcγR), employed by the virus to evade the effector consequences of anti-HCMV antibody binding, has differential affinity for IgG1 proteins expressing distinct GM alleles. Specifically, the HCMV FcγR encoded by the viral *TRL11/IRL11* gene has significantly higher affinity for IgG1 proteins expressing the GM 3+,1-,2- allotypes than for those expressing the allelic GM 17+,1+,2+ allotypes.¹⁷ These observations led us to hypothesize that these GM alleles might represent risk or protective factors, respectively, for the development of HCMV-induced glioma.¹⁸ In the present work,

*Correspondence to: Janardan P Pandey; Email: pandeyj@muscc.edu

Submitted: 12/31/2013; Revised: 03/11/2014; Accepted: 03/20/2014; Published Online: 05/23/2014

Citation: Pandey JP, Kaur N, Costa S, Amorim J, Nabico R, Linhares P, Vaz R, Viana-Pereira M, Reis RM. Immunoglobulin gene allotypes implicated in glioma risk. *Oncolimmunology* 2014; 3:e28609; <http://dx.doi.org/10.4161/onci.28609>

we applied a case-control design to test the hypothesis that subjects expressing the GM 3 allele would be at higher risk of developing glioma.

Results

The demographics of our study population, including patient clinicopathologic features, are described in Table 1. Results of multivariate regression analysis to assess associations between GM genotypes or allele frequencies and glioma risk among the various histologic subgroups are presented in Table 2. In comparison to subjects who were homozygous for the GM 17 allele, the GM 3 homozygotes were over twice as likely (OR = 2.82, CI 1.05–7.59), and the GM 3/17 heterozygotes were over three times as likely (OR = 3.13, CI 1.15–8.52), to develop glioma, irrespective of disease subtype (all grades). Similarly, in comparison to GM 17/17 homozygotes, GM 3/17 heterozygotes were over three times as likely to develop high-grade astrocytoma (OR = 3.37, CI 1.05–10.87). Likewise, when comparing the combined GM 3/3+3/17 (i.e. GM 3-carriers) genotypes to the

GM 17/17 genotypes, the presence of the GM 3 allele conferred a significantly increased risk for glioma (OR = 2.95, CI 1.13–7.73) and high-grade astrocytoma (OR = 3.11, CI 1.01–9.62). Similar results were obtained when assessing the allele frequencies. It was shown that the GM 3 allele was a risk factor for glioma (OR = 1.52, CI 1.10–2.11) and high-grade astrocytoma (OR = 1.49, CI 1.03–2.15), when compared with the reference GM 17 variant. No significant associations were found between GM genotypes or GM alleles and the risk of oligodendroglioma.

We also applied a multivariate Cox proportional hazard model (adjusted for patient age and sex) to investigate the association between GM polymorphism and overall survival. Glioblastoma patients for which follow up information was available who carried the GM 17/17 genotype (n = 4, 2 deaths) had a longer median overall survival than patients carrying the GM 3/17 (n = 18, 14 deaths) or GM 3/3 (n = 23, 17 deaths) genotypes with a median survival of 35 mo (range, 2–38) versus 16 mo (range, 3–52), respectively. However, the difference was not statistically significant (HR = 2.78; 95% CI, 0.61–12.65, *P* = 0.19), probably due to limited sample size in analysis (Fig. 1). (The survival curves for the three genotypes separately are presented in Figure S1.)

Table 1. Clinicopathologic features of gliomas and controls

Groups (WHO grade)	N	Age years (mean ± SD)	Male/female ratio
Controls	133	36–85 (54.80 ± 9.39)	1.6
Glioma (all grades)	120	20–83 (56.38 ± 12.55)	1.6
Astrocytoma (all grades)	97	22–83 (57.55 ± 12.21)	1.9
Oligodendroglioma (all grades)	23	20–78 (51.48 ± 13.08)	0.8
Astrocytoma (grades II–IV)	95	22–83 (57.67 ± 12.04)	1.9
Astrocytoma (grades III–IV)	90	22–83 (58.21 ± 11.80)	2.0

Table 2. Multivariate logistic regression analysis of associations between GM variants and risk of glioma

	Control	Glioma (All Grades)	OR (95% CI) ¹	Astrocytoma (Grades III–IV)	OR (95% CI) ¹	Oligodendroglioma (All Grades)	OR (95% CI) ¹
Genotypes*							
GM 17/17	18	6	-	4	-	2	
GM 3/3	64	61	2.82 (1.05–7.59) <i>P</i> = 0.041	46	2.91 (0.91–9.28) <i>P</i> = 0.071	10	1.67 (0.32–8.53) <i>P</i> = 0.55
GM 3/17	51	53	3.13 (1.15–8.52) <i>P</i> = 0.026	40	3.37 (1.05–10.87) <i>P</i> = 0.042	11	2.09 (0.41–10.61) <i>P</i> = 0.37
GM 3/3+3/17	115	114	2.95 (1.13–7.73) <i>P</i> = 0.027	86	3.11 (1.01–9.62) <i>P</i> = 0.049	21	1.86 (0.39–8.88) <i>P</i> = 0.43
	133	120		90		23	
Alleles**							
17	0.327	0.271	-	0.267	-	0.326	
3	0.673	0.729	1.52 (1.10–2.11) <i>P</i> = 0.012	0.733	1.49 (1.03–2.15) <i>P</i> = 0.033	0.674	1.15 (0.65–2.05) <i>P</i> = 0.637

Significance of associations between GM genotypes or alleles and risk of glioma in various histologic subgroups. *Reference genotype GM 17/17; **Reference allele 17; regression models included age and sex as covariates. 1OR (95% CI)—Odds ratio with 95% confidence intervals.

Discussion

Results presented here show that carriers of the GM 3 allele of IgG1 have 3-fold higher risk of high-grade glioma than non-carriers. Potential underlying mechanisms could involve a direct contribution of GM alleles to immunity to self and non-self antigens relevant to gliomagenesis. Alternatively, it is possible that there may be another locus affecting glioma susceptibility that is distinct from GM on chromosome 14 and whose alleles are in significant linkage disequilibrium with those of the GM loci. This putative linkage disequilibrium could give rise to the associations observed. The most relevant among the self-antigens, immunity to which is influenced by GM alleles, are human epidermal growth factor receptor 1 (HER1/EGFR) and HER2. These tumor-associated antigens are aberrantly expressed in gliomas^{19,20} and we have previously shown that GM alleles epistatically interact with FcγRIIIa alleles expressed on natural killer cells and mediate antibody-dependent cellular cytotoxicity of EGFR- and HER2-overexpressing cancer cells.²¹ Though not yet investigated in glioma, antibody responses to HER2 are significantly associated with GM alleles in patients with breast cancer.^{22,23} This may hold for patients with glioma as well.

The most relevant among the non-self factors, immunity to which is influenced by GM alleles, is HCMV. As mentioned before, the current consensus in the glioma field is that HCMV is an active promoter or oncogenic modulator of gliomagenesis, not merely a bystander.¹⁶ GM alleles could influence HCMV-glioma association by influencing antibody responsiveness to viral epitopes and further by modulating the viral immunoevasion strategies. In the inflammatory autoimmune disease scleroderma (systemic sclerosis), HCMV appears to accelerate the development of disparate disease pathologies^{24–26} and, of particular importance, we have found a highly significant association between anti-HCMV IgG antibody responses and the GM 3 and GM 17 alleles expressed on IgG1 in these patients.²⁷ Similar studies evaluating correlations between the presence of particular GM allelic variants and HCMV antibodies should be conducted for glioma patients.

GM allotypes are expressed in the constant region of γ chains. How could these constant-region determinants influence immune responsiveness thought to be exclusively associated with the variable-region genes? Recent investigations have challenged this central tenet of immunology. Several studies have shown that structural variation in the constant region affects the expression of certain idiotypes and causes variation in the specificity of variable-region-identical immunoglobulin molecules.²⁸ It is especially noteworthy that amino acid sequence polymorphism in the CH1 domain of the γ1 chain—where the allelic determinants GM 3 and GM 17 are located—has been shown to modulate the kinetic competence of antigen binding sites.²⁹ Thus, amino acid substitutions associated with GM allotypes cause structural changes in the constant region, which could translate to structural constraints (conformation) imposed on the variable region, resulting in variation in antibody specificity and affinity.

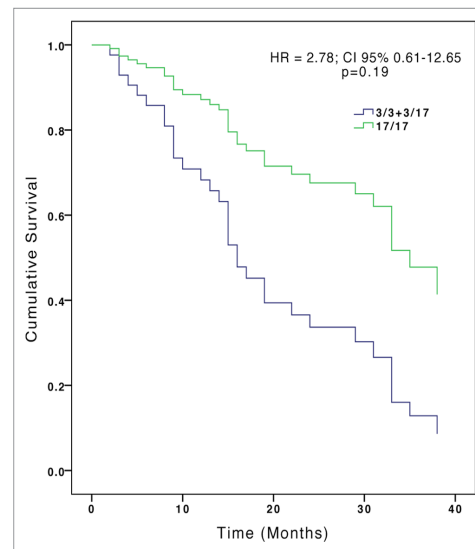


Figure 1. Correlation analysis of overall survival of glioblastoma patients with GM allotypes. Multivariate Cox proportional hazard model regression with respect to γ marker (GM) genotypes in the sub-groups of GM 17/17 (n = 4) and GM 3/3 + GM 3/17 (n = 38) and glioblastoma patient survival. Hazard ratio (HR) = 2.78; 95% confidence interval (CI) 0.61–12.65, P = 0.19.

As mentioned above, GM alleles modulate one of HCMV's immunoevasion strategies. The HCMV *TRL111/IRL111*-encoded FcγR—which enables the virus to evade the effector consequences of antibody binding, such as antibody-dependent cellular cytotoxicity, complement-dependent neutralization, and phagocytosis—discriminates between IgG1 proteins expressing GM 3 and GM 17 alleles. The HCMV-encoded FcγR has significantly higher affinity for IgG1 proteins expressing the GM 3+,1-,2- allotypes than for those expressing the allelic GM 17+,1+,2+ allotypes.¹⁷ It naturally follows that individuals expressing the relatively higher affinity GM 3+,1-,2- alleles would be more likely to have their Fc domains scavenged, thereby reducing their immunological competence to eliminate the virus through Fc-mediated effector mechanisms. Therefore, one would anticipate that decreased immunity against HCMV would be conducive to glioma development, such that the frequency of GM 3+,1-,2- alleles would be expected to be higher in glioma patients than in healthy controls. In fact, our findings are fully consistent with this prediction.

If GM genes are risk factors for glioma, as shown here, why have they not been detected by the genome-wide association studies (GWAS) of this malignancy?³⁰ A likely reason underlying this deficiency may be the absence of these genes in many commonly used genotyping platforms. The *IGHG* gene segments harboring GM alleles are highly homologous (> 95%) and apparently not amenable to the high throughput genotyping technology used

in GWAS. Furthermore, because these genes were not typed in either the HapMap or 1000 Genomes projects, they cannot be imputed or tagged via linkage disequilibrium by single nucleotide polymorphisms (SNPs) included in the genotyping platforms. This underscores the necessity for the candidate gene approach to prospectively investigate the causal role for the *IGHG* gene complex, particularly the GM alleles, in the immunopathology of glioma.

Future studies involving GM allotypes and disease risk may also shed light on the reasons behind the racial differences in the prevalence of glioma. Age-adjusted glioma rates are considerably higher in Caucasians than in people of African descent.² In these regards, it is potentially highly relevant that the GM 3 allele, implicated in the risk of glioma in our study here, is either absent, or extremely rare, in people with unmixed African ancestry.¹¹

Future studies should also consider examining the interactive effects of particular candidate genes in gliomagenesis. Genes do not act in isolation and there is preponderant evidence that epistasis—modification of the action of one gene by the action of one, or more, other genes—plays a significant role in human disease processes.^{31–33} With this in mind, *IGHG* (GM allotypes) and *HLA* are excellent candidate genes for epistatic investigations in glioma, as both are targets of HCMV immunoevasion strategies^{17,34} and have been shown to contribute to glioma risk, GM alleles in the present investigation and HLA alleles in previous studies.^{1,2}

To our knowledge, this is the first report implicating GM genes in susceptibility to glioma, a devastating and often lethal malignancy. Replication, using a larger, independent cohort, is needed to validate the results presented here.

Material and Methods

Glioma patients and controls

The study population has been described in detail elsewhere.⁶ All samples with available DNA isolated from peripheral blood lymphocytes were included in this work. Briefly, 120 glioma patients were recruited at Hospital de Braga, Braga and Hospital S. João, Porto, Portugal. Controls consisted of 133 cancer-free random blood donors. Both patients and controls were Caucasians. The study protocol was approved by the Institutional Ethical Committees. Peripheral blood samples from patients and normal controls were collected after informed consent, according to the Helsinki Declaration.

Quantitative PCR-based GM genotyping

At position 214 of the $\gamma 1$ chain, an arginine residue characterizes the GM 3 allele and a lysine characterizes the GM 17 allele. This corresponds to a G to A nucleotide substitution in the $\gamma 1$ gene. We used a pre-designed TaqMan® genotyping assay targeting these variants from Applied Biosystems Inc.,

according to manufacturer's instructions and employing the following primers and probes: forward primer (5' CCCAGACCTA CATCTGCAAC GTGA-3'); reverse primer (5' CTGCCCTGGA CTGGGACTGC AT-3'); reporter 1 (GM 17-specific) (VIC-CTCTCACCAA CTTTCTTGT-NFQ); reporter 2 (GM 3-specific) (FAM-CTCTCACCAA CTCTCTTGT-NFQ)

Statistical analyses to evaluate associations between glioma and GM genotype

Genotype frequencies were in Hardy–Weinberg equilibrium in both groups. To evaluate the effect of an IgG1 GM variant on the risk of glioma, odds ratios (OR) and 95% confidence intervals (95% CI) were estimated by unconditional multivariate logistic regression analysis adjusted for potential confounding variables (i.e., patient age and sex). We estimated ORs of glioma risk associated with the GM 3/3 or GM 3/17 genotype relative to that of GM 17/17 used as reference. We also analyzed a model in which the combined GM 3/3+3/17 was compared with the genotype GM 17/17 (reference). Additionally, the allelic frequency of 3 and 17 alleles was estimated, as well as the ORs associated with risk of glioma. Associations between GM variants and patient survival were assessed using a multivariate Cox regression model adjusted for patient age, and sex. In this analysis, the genotypes and patient survival for GM 3/17+GM 3/3 were compared with those of GM 17/17 patients, again used as a reference standard. Our rationale for choosing the less frequent (GM 17) allele as reference is that our hypothesis is based on the fact that people expressing the GM 3 variant have a reduced immunological competence to eliminate HCMV and, as such, have an increased risk to develop glioma. Patients were followed until death or loss to follow-up. Statistical significance was defined as $P < 0.05$. All reported P values are 2-sided. Data analysis was performed using SPSS 20.0 software (SPSS, Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/oncoimmunology/article/28609

Acknowledgments

This work was supported in part by the US. National Institute of Neurological Disorders and Stroke and by Fundação a Ciência e Tecnologia, Portugal (PTDC/SAU-ONC/115513/2009). The authors thank the Immunochemotherapy Department of Hospital S. Marcos, and Clínica Laboratorial Dr. Edgar Botelho Moniz, S. Tirso, Portugal, for their helpful assistance in the management of controls, and to Monica Ferreira from ICVS for her valuable assistance in the management of cases and controls.

References

- Tang J, Shao W, Dorak MT, Li Y, Mücke R, Lobashovsky E, Wiencke JK, Wrensch M, Kaslow RA, Cobbs CS. Positive and negative associations of human leukocyte antigen variants with the onset and prognosis of adult glioblastoma multiforme. *Cancer Epidemiol Biomarkers Prev* 2005; 14:2040-4; PMID:16103458; <http://dx.doi.org/10.1158/1055-9965.EPI-05-0136>
- Song W, Ruder AM, Hu L, Li Y, Ni R, Shao W, Kaslow RA, Butler M, Tang J. Genetic epidemiology of glioblastoma multiforme: confirmatory and new findings from analyses of human leukocyte antigen alleles and motifs. *PLoS One* 2009; 4:e7157; PMID:19774073; <http://dx.doi.org/10.1371/journal.pone.0007157>
- Schwartzbaum JA, Xiao Y, Liu Y, Tsavachidis S, Berger MS, Bondy ML, Chang JS, Chang SM, Decker PA, Ding B, et al. Inherited variation in immune genes and pathways and glioblastoma risk. *Carcinogenesis* 2010; 31:1770-7; PMID:20668009; <http://dx.doi.org/10.1093/carcin/bgq152>
- Amirani ES, Scheurer ME, Liu Y, D'Amelio AM Jr., Houlston RS, Ertel CJ, Shete S, Swerdlow AJ, Schoemaker MJ, McKinney PA, et al. A novel approach to exploring potential interactions among single-nucleotide polymorphisms of inflammation genes in gliomagenesis: an exploratory case-only study. *Cancer Epidemiol Biomarkers Prev* 2011; 20:1683-9; PMID:21724854; <http://dx.doi.org/10.1158/1055-9965.EPI-11-0203>
- Rajaraman P, Melin BS, Wang Z, McKean-Cowdin R, Michaud DS, Wang SS, Bondy M, Houlston R, Jenkins RB, Wrensch M, et al. Genome-wide association study of glioma and meta-analysis. *Hum Genet* 2012; 131:1877-88; PMID:22886559; <http://dx.doi.org/10.1007/s00439-012-212-0>
- Costa BM, Viana-Pereira M, Fernandes R, Costa S, Linhares P, Vaz R, Pinheiro C, Lima J, Soares P, Silva A, et al. Impact of EGFR genetic variants on glioma risk and patient outcome. *Cancer Epidemiol Biomarkers Prev* 2011; 20:2610-7; PMID:21960689; <http://dx.doi.org/10.1158/1055-9965.EPI-11-0340>
- Lima-Ramos V, Pacheco-Figueiredo L, Costa S, Pardal F, Silva A, Amorim J, Lopes JM, Reis RM. TP53 codon 72 polymorphism in susceptibility, overall survival, and adjuvant therapy response of gliomas. *Cancer Genet Cytogenet* 2008; 180:14-9; PMID:18068527; <http://dx.doi.org/10.1016/j.cancergencyto.2007.08.019>
- Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A, Pardal F, Suriano G, Machado JC, Lopes JM, et al. Association between functional EGF+61 polymorphism and glioma risk. *Clin Cancer Res* 2007; 13:2621-6; PMID:17473192; <http://dx.doi.org/10.1158/1078-0432.CCR-06-2606>
- Kohanbash G, Ishikawa E, Fujita M, Ikeura M, McKaveney K, Zhu J, Sakaki M, Sarkar SN, Okada H. Differential activity of interferon- α promoter is regulated by Ocr-1 and a SNP that dictates prognosis of glioma. *Oncoimmunology* 2012; 1:487-92; PMID:22754767; <http://dx.doi.org/10.4161/onci.19964>
- Kohanbash G, McKaveney K, Sakaki M, Ueda R, Mintz AH, Amankulor N, Fujita M, Ohlfest JR, Okada H. GM-CSF promotes the immunosuppressive activity of glioma-infiltrating myeloid cells through interleukin-4 receptor- α . *Cancer Res* 2013; 73:6413-23; PMID:24030977; <http://dx.doi.org/10.1158/0008-5472.CAN-12-4124>
- Pandey JP, Li Z. The forgotten tale of immunoglobulin allotypes in cancer risk and treatment. *Exp Hematol Oncol* 2013; 2:6; PMID:23425356; <http://dx.doi.org/10.1186/2162-3619-2-6>
- Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, Nabors LB, Cobbs CG, Britt WJ. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res* 2002; 62:3347-50; PMID:12067971
- Mitchell DA, Xie W, Schmitling R, Learn C, Friedman A, McLendon RE, Sampson JH. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. *Neuro Oncol* 2008; 10:10-8; PMID:17951512; <http://dx.doi.org/10.1215/15228517-2007-035>
- Scheurer ME, Bondy ML, Aldape KD, Albrecht T, El-Zein R. Detection of human cytomegalovirus in different histological types of gliomas. *Acta Neuropathol* 2008; 116:79-86; PMID:18351367; <http://dx.doi.org/10.1007/s00401-008-0359-1>
- Cobbs CS. Evolving evidence implicates cytomegalovirus as a promoter of malignant glioma pathogenesis. *Herpesviridae* 2011; 2:10; PMID:22030012; <http://dx.doi.org/10.1186/2042-4280-2-10>
- Dziurzynski K, Chang SM, Heimberger AB, Kaleja RF, McGregor Dallas SR, Smit M, Sorocanu L, Cobbs CS; HCMV and Gliomas Symposium. Consensus on the role of human cytomegalovirus in glioblastoma. *Neuro Oncol* 2012; 14:246-55; PMID:22319219; <http://dx.doi.org/10.1093/neuonc/nor227>
- Nambodiri AM, Pandey JP. The human cytomegalovirus *TRL111/IRL1*-encoded Fc γ R binds differentially to allelic variants of immunoglobulin G1. *Arch Virol* 2011; 156:907-10; PMID:21311920; <http://dx.doi.org/10.1007/s00705-011-0937-8>
- Pandey JP. Genetic and viral etiology of glioblastoma—a unifying hypothesis. *Cancer Epidemiol Biomarkers Prev* 2011; 20:1061-3; PMID:21586624; <http://dx.doi.org/10.1158/1055-9965.EPI-11-0247>
- Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS, Vogelstein B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 1992; 89:2965-9; PMID:1557402; <http://dx.doi.org/10.1073/pnas.89.7.2965>
- Kristt DA, Yarden Y. Differences between phosphotyrosine accumulation and Neu/ ErbB-2 receptor expression in astrocytic proliferative processes. Implications for glial oncogenesis. *Cancer* 1996; 78:1272-83; PMID:8826951; [http://dx.doi.org/10.1002/\(SICI\)1097-0142\(19960915\)78:6<1272::AID-CNCR16>3.0.CO;2-Y](http://dx.doi.org/10.1002/(SICI)1097-0142(19960915)78:6<1272::AID-CNCR16>3.0.CO;2-Y)
- Nambodiri AM, Pandey JP. Differential inhibition of trastuzumab- and cetuximab-induced cytotoxicity of cancer cells by immunoglobulin G1 expressing different GM allotypes. *Clin Exp Immunol* 2011; 166:361-5; PMID:22059994; <http://dx.doi.org/10.1111/j.1365-2249.2011.04477.x>
- Pandey JP, Nambodiri AM, Kurtenkov O, Nietert PJ. Genetic regulation of antibody responses to human epidermal growth factor receptor 2 in breast cancer. *Hum Immunol* 2010; 71:1124-7; PMID:20736037; <http://dx.doi.org/10.1016/j.humimm.2010.08.009>
- Pandey JP, Nambodiri AM, Kistner-Griffin E, Iwasaki M, Kasuga Y, Hamada GS, Tsugane S. Racially restricted contribution of immunoglobulin Fc γ and Fc γ receptor genotypes to humoral immunity to human epidermal growth factor receptor 2 in breast cancer. *Clin Exp Immunol* 2013; 171:273-7; PMID:23379433; <http://dx.doi.org/10.1111/cei.12018>
- Pandey JP, LeRoy EC. Human cytomegalovirus and the vasculopathies of autoimmune diseases (especially scleroderma), allograft rejection, and coronary stenosis. *Arthritis Rheum* 1998; 41:10-5; PMID:9433864; [http://dx.doi.org/10.1002/1529-0131\(199801\)41:1<10::AID-ART2>3.0.CO;2-P](http://dx.doi.org/10.1002/1529-0131(199801)41:1<10::AID-ART2>3.0.CO;2-P)
- Lunardi C, Bason C, Navone R, Millo E, Damonte G, Corrocher R, Puccetti A. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. *Nat Med* 2000; 6:1183-6; PMID:11017152; <http://dx.doi.org/10.1038/80533>
- Lunardi C, Dolcino M, Peterlana D, Bason C, Navone R, Tamassia N, Beri R, Corrocher R, Puccetti A. Antibodies against human cytomegalovirus in the pathogenesis of systemic sclerosis: a gene array approach. *PLoS Med* 2006; 3:e2; PMID:16318412; <http://dx.doi.org/10.1371/journal.pmed.0030002>
- Pandey JP. Immunoglobulin GM genes and IgG antibodies to cytomegalovirus in patients with systemic sclerosis. *Clin Exp Rheumatol* 2004; 22(Suppl 33):S35-7; PMID:15344596
- Casadevall A, Pirofski L-A. A new synthesis for antibody-mediated immunity. *Nat Immunol* 2012; 13:21-8; PMID:22179281; <http://dx.doi.org/10.1038/ni.2184>
- Torres M, Fernández-Fuentes N, Fiser A, Casadevall A. The immunoglobulin heavy chain constant region affects kinetic and thermodynamic parameters of antibody variable region interactions with antigen. *J Biol Chem* 2007; 282:13917-27; PMID:17353196; <http://dx.doi.org/10.1074/jbc.M700661200>
- Enciso-Mora V, Hosking FJ, Kinnerley B, Wang Y, Shete S, Zelenika D, Broderick P, Idbaih A, Delattre JY, Hoang-Xuan K, et al. Deciphering the 8q24.21 association for glioma. *Hum Mol Genet* 2013; 22:2293-302; PMID:23399484; <http://dx.doi.org/10.1093/hmg/ddt063>
- Moore JH. The ubiquitous nature of epistasis in determining susceptibility to common human diseases. *Hum Hered* 2003; 56:73-82; PMID:14614241; <http://dx.doi.org/10.1159/000073735>
- Phillips PC. Epistasis—the essential role of gene interactions in the structure and evolution of genetic systems. *Nat Rev Genet* 2008; 9:855-67; PMID:18852697; <http://dx.doi.org/10.1038/nrg2452>
- Lehner B. Molecular mechanisms of epistasis within and between genes. *Trends Genet* 2011; 27:323-31; PMID:21684621; <http://dx.doi.org/10.1016/j.tig.2011.05.007>
- Jackson SE, Mason GM, Wills MR. Human cytomegalovirus immunity and immune evasion. *Virus Res* 2011; 157:151-60; PMID:21056604; <http://dx.doi.org/10.1016/j.virusres.2010.10.031>

4 IMPACT OF TGF- β 1-509C/T AND 869T/C POLYMORPHISMS ON GLIOMA RISK AND PATIENT PROGNOSIS

Joana Vieira de Castro,^{1,2} Céline S. Gonçalves,^{1,2} Sandra Costa,^{1,2} Paulo Linhares,³ Rui Vaz,³ Ricardo Nabiço,⁴ Júlia Amorim,⁴ Marta Viana-Pereira,^{1,2} Rui M. Reis,^{1,2,5} Bruno M. Costa^{1,2}

¹ Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; ² ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal; ³ Department of Neurosurgery, Hospital São João, Alameda Professor Hernâni Monteiro, 4202-451 Porto, Portugal; ⁴ Department of Oncology, Hospital de Braga, Sete Fontes - São Victor, 4710-243 Braga, Portugal; ⁵ Molecular Oncology Research Center, Barretos Cancer Hospital, Rua Antenor Duarte Vilela, 1331 - Doutor Paulo Prata, Barretos, SP 14780-000, Brazil.

Impact of *TGF-β1* -509C/T and 869T/C polymorphisms on glioma risk and patient prognosis

Joana Vieira de Castro^{1,2} · Céline S. Gonçalves^{1,2} · Sandra Costa^{1,2} · Paulo Linhares³ · Rui Vaz³ · Ricardo Nabico⁴ · Júlia Amorim⁴ · Marta Viana-Pereira^{1,2} · Rui M. Reis^{1,2,5} · Bruno M. Costa^{1,2}

Received: 15 January 2015 / Accepted: 15 March 2015
© International Society of Oncology and BioMarkers (ISOBM) 2015

Abstract Transforming growth factor beta (TGF-β) plays an important role in carcinogenesis. Two polymorphisms in the *TGF-β1* gene (-509C/T and 869T/C) were described to influence susceptibility to gastric and breast cancers. The 869T/C polymorphism was also associated with overall survival in breast cancer patients. In the present study, we investigated the relevance of these *TGF-β1* polymorphism in glioma risk and prognosis. A case-control study that included 114 glioma patients and 138 cancer-free controls was performed. Single nucleotide polymorphisms (SNPs) were evaluated by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Univariate and multivariate logistic regression analyses were used to calculate odds ratio (OR) and 95 % confidence intervals (95 % CI). The influence of *TGF-β1*-509C/T and 869T/C polymorphisms on glioma patient survival was evaluated by a Cox regression model

adjusted for patients' age and sex and represented in Kaplan-Meier curves. Our results demonstrated that *TGF-β1* gene polymorphisms -509C/T and 869T/C are not significantly associated with glioma risk. Survival analyses showed that the homozygous -509TT genotype associates with longer overall survival of glioblastoma (GBM) patients when compared with patients carrying CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; $p=0.036$). In addition, the homozygous 869CC genotype is associated with increased overall survival of GBM patients when compared with 869TT+TC genotypes (OR, 2.62; 95 % CI, 1.11–6.17; $p=0.027$). In conclusion, this study suggests that *TGF-β1*-509C/T and 869T/C polymorphisms are not significantly associated with risk for developing gliomas but may be relevant prognostic biomarkers in GBM patients.

Keywords Glioma · Glioblastoma · Transforming growth factor beta 1 · Single nucleotide polymorphisms · Risk · Prognosis

✉ Bruno M. Costa
bfmcosta@ecsaude.uminho.pt

¹ Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

² ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

³ Department of Neurosurgery, Hospital São João, Alameda Professor Hernâni Monteiro, 4202-451 Porto, Portugal

⁴ Department of Oncology, Hospital de Braga, Sete Fontes – São Victor, 4710-243 Braga, Portugal

⁵ Molecular Oncology Research Center, Barretos Cancer Hospital, Rua Antenor Duarte Vilela, 1331 - Doutor Paulo Prata, Barretos, SP 14780-000, Brazil

Introduction

During the last decades, the incidence and mortality of brain tumors have increased in most developed countries, mainly in the older age groups, with a slightly higher incidence in men than in women [1]. Gliomas, the most common primary tumors of the central nervous system (CNS), account for almost 80 % of brain malignancies [2]. According to their histological characteristics, these tumors can be divided into four main subgroups: astrocytomas, oligodendrogliomas, oligoastrocytomas, and ependymomas (the less common). Glioma tumors can also be divided into four grades of

malignancy according to the World Health Organization (WHO) classification, being glioblastoma (GBM) the most common and biologically aggressive glioma type (grade 4) [3, 4]. Despite the advances in the field of neuro-oncology, the prognosis of glioma patients remains very poor [5], particularly for patients with GBM [6]. Few factors have been associated with increased glioma risk, including hereditary syndromes, such as Li-Fraumeni and Turcot syndromes, neurofibromatosis (type 1 and type 2) and tuberous sclerosis complex [7, 8], familial aggregation [7, 9], and exposure to high doses of ionizing radiation [7, 10, 11]. Some genome-wide association studies have showed that single nucleotide polymorphisms (SNPs) are associated with glioma susceptibility [12, 13]. However, other factors that may contribute to glioma susceptibility require additional investigation.

The transforming growth factor beta (TGF- β), a multifunctional cytokine, is involved in the regulation of several immunomodulatory processes that play a key role in numerous cellular processes, such as proliferation, differentiation, apoptosis, angiogenesis, tumor progression, and extracellular matrix production [14]. TGF- β has three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3. These three isoforms bind and activate a membrane receptor serine/threonine complex (type I TGF- β RI and type II TGF- β RII). The intracellular signaling is initiated when TGF- β RII phosphorylates TGF- β RI, which in turn phosphorylates the transcription factors Smad2 or Smad3 that consequently bind Smad4. This complex is translocated from the cytoplasm to the nucleus, resulting in the transcriptional activation of TGF- β responsive genes that ultimately mediate the effects of TGF- β at the cellular level [15]. Deregulation of TGF- β signaling has been implicated in cancer, where TGF- β has been demonstrated to have a dual role. It may act as a strong inhibitor of proliferation of normal astrocytes and epithelial cells, being considered a tumor suppressor factor, but in some tumor types, including high-grade glioma, TGF- β acts as an oncogenic factor contributing to cell growth and invasion and decreases host immune responses against tumor [16]. It was also demonstrated that TGF- β activity confers poor prognosis in glioma patients [17, 18]. Several studies have identified *TGF- β 1* as a predictive cancer biomarker, particularly focusing on *TGF- β 1* genetic polymorphisms [19–22]. In fact, it was demonstrated that polymorphisms in this gene contribute to breast and gastric cancers susceptibility [19, 23]. Additionally, studies demonstrated an association of *TGF- β 1* 869T/C polymorphism with overall survival of breast cancer patients [24, 25]. The *TGF- β 1* gene is located on chromosome 19q13, and two common polymorphisms of the *TGF- β 1* gene have been extensively studied, the -509C/T (rs1800469) and the 869T/C (rs1800470, previously known as rs1982073; T29C and Leu10Pro) [22, 26, 27]. The -509C/T polymorphism is located in the promoter region of *TGF- β 1* gene, which may potentially regulate *TGF- β 1*

transcription. The 869T/C polymorphism is located in exon 1 and could lead to a leucine-to-proline substitution at codon 10 [21, 26]. Some studies demonstrated that the -509T allele is associated with an increased transcriptional activity as compared to -509C allele [28], which leads to a higher serum concentration of TGF- β 1 among TT homozygotes than in the CT heterozygotes [29]. Similarly, the 869C allele was associated with high serum concentrations of TGF- β 1 [19, 30]. Moreover, some studies showed that -509C/T and 869T/C *TGF- β 1* polymorphisms were able to affect TGF- β 1 protein expression [31, 32]. Importantly, the circulating levels of this cytokine have been associated with cancer [33–35]. The relevance of *TGF- β 1* polymorphisms has not been reported in gliomas. Thus, the aim of this case-control study was to investigate the relevance of *TGF- β 1*-509C/T and 869T/C polymorphisms in glioma susceptibility and how specific polymorphic variants may influence the prognosis of patients.

Methods

Study population

In this case-control study, we enrolled 114 glioma patients from Portugal (Hospital of Braga, Braga, and Hospital São João, Porto) diagnosed between 2004 and 2013. The peripheral blood from these subjects was collected. Tumors were classified according to WHO [3], and clinico-pathological features are summarized in Table 1. The control group was randomly selected from blood donors at Hospital of Braga, and it included 138 cancer-free individuals. All subjects were of Caucasian ethnic background. The procedures followed in the present study were in accordance with institutional ethical standards.

Genotyping

Genomic DNA from glioma cases and controls was extracted from peripheral blood leukocytes by proteinase K/chloroform/isopropanol treatment [36]. The purified DNA was used to determine the genotypes of both polymorphisms, using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) methods. The PCR primers for -509C/T polymorphism were 5'-CCCGGCTCCATTTCACGGTG-3' (forward) and 5'-GGTCAACCAGAGAAAGAGGAC-3' (reverse), and for the 869T/C polymorphism were 5'-CCTCCCCACCACACCAG-3' (forward) and 5'-CCGCAGCTTGACAGG-3' (reverse). The PCR was performed in a total volume of 25 μ l containing 50 ng of DNA, 0.5 U of KAPA Taq DNA polymerase (GRiSP), 1 $\times$ KAPA Taq Buffer A containing MgCl₂, 0.2 mM dNTP mix, and 0.8 μ M of each primer. For the -509C/T polymorphism, the DNA was initially

Table 1 Clinico-pathological features of gliomas and controls

Groups (WHO grade)	Number of cases	Age, year (mean±SD)	Male/female ratio
Controls	138	40.9±12.1	1.2
Gliomas (2–4)	114	58.3±12.9	1.7
Astrocytomas (2–4)	97	58.9±12.7	2.2
Astrocytomas (2–3)	8	52.3±13.3	1
Diffuse astrocytomas (2)	5	54.8±13.8	0.67
Anaplastic astrocytomas (3)	2	55.0±9.9	1
Gliosarcomas (4)	4	61.3±9.1	All males
Glioblastomas (4)	85	59.5±12.7	2.1
Oligodendrogliomas (2–3)	16	53.1±12.3	0.45
Oligodendrogliomas (2)	4	46.3±9.7	All females
Anaplastic Oligodendrogliomas (3)	10	54.7±12.9	0.67

denatured at 95 °C for 7 min, followed by 11 cycles of 95 °C for 30 s, 66–61 °C for 30 s, and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min. The PCR was finished by a final extension cycle at 72 °C for 8 min. Regarding 869T/C polymorphism, the PCR cycle conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 9 cycles of 95 °C for 30 s, 68–64 °C for 30 s, and 72 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. Finally, the PCR was completed by a final extension cycle at 72 °C for 8 min. After confirmation of an amplified fragment of the expected size (808 bp for -509C/T and 235 bp for 869T/C)

on 2 % agarose gel, 8–12 µL of PCR products were digested overnight at 37 °C with the appropriate restriction enzymes. For the -509C/T polymorphism, 10 U of restriction enzyme Bsu36I (New England Biolabs) was used, and for the 869T/C, 5 U of the restriction enzyme MspAII (Fermentas) was applied. The DNA fragments were resolved on 2 % agarose gel for -509C/T polymorphism and 4 % agarose gel for 869T/C polymorphism and were detected by Greensafe Premium staining (Nzytech). For -509C/T polymorphism, the PCR product (808 bp) with C allele was digested into two fragments (617 and 191 bp), whereas the PCR product with T allele was not digested by Bsu36I. For 869T/C polymorphism,

Table 2 Univariate analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

Polymorphism	Control	Glioma (WHO grades 2–4)	OR (95 % CI) ^a	Glioblastoma (WHO grade 4)	OR (95 % CI) ^a
<i>TGF-β1</i> -509C/T					
Genotypes					
TT	22	18	–	16	–
CC	54	42	0.95 (0.45–1.98)	28	0.71 (0.32–1.57)
CT	62	54	1.07 (0.52–2.19)	41	0.91 (0.43–1.94)
CC+CT	116	96	1.01 (0.51–2.00)	69	0.82 (0.40–1.66)
Alleles					
T	0.384	0.395	–	0.429	–
C	0.616	0.605	0.97 (0.68–1.39)	0.571	0.85 (0.57–1.25)
<i>TGF-β1</i> 869T/C					
Genotypes					
CC	26	19	–	17	–
TT	48	42	1.20 (0.58–2.47)	30	0.96 (0.45–2.05)
TC	64	53	1.13 (0.57–2.27)	38	0.91 (0.44–1.89)
TT+TC	112	95	1.16 (0.61–2.23)	68	0.93 (0.47–1.84)
Alleles					
C	0.420	0.399	–	0.424	–
T	0.580	0.601	1.09 (0.76–1.56)	0.576	1.10 (0.75–1.64)

^a Odds ratio (OR) with 95 % confidence intervals (CI)

the PCR product (235 bp) with T allele was digested into four fragments (103, 67, 40, and 25 bp), and the PCR product with C allele was digested into five fragments (91, 67, 40, 25, and 12 bp).

Statistical analysis

Data analysis was performed using SPSS 22.0 software (SPSS, Inc.). Differences in allele and genotype frequencies were compared between glioma patients and cancer-free controls by the chi-square test, and the frequency distribution of age and sex was compared between glioma patients and cancer-free controls by the nonparametric Wilcoxon-Mann Whitney test. Additionally, the chi-square test was used to verify that the observed allele distribution, in the control group, was in Hardy-Weinberg equilibrium. Odds ratio (OR) and 95 % confidence intervals (95 % CI) were estimated by univariate and multivariate logistic regression analyses, adjusted for patients' age (as a continuous variable) and sex, to assess the risk for each glioma type conferred by a particular

allele and genotype of each polymorphism. Patient survival curves were assessed by the Kaplan-Meier method for GBM. A Cox regression model adjusted for patients' age (as a continuous variable) and sex was applied to evaluate the effect of the *TGF-β1* genotypes on overall survival. Statistical significance was considered for *p* values <0.05.

Results

The clinico-pathological features of the controls and cases are summarized in Table 1. For both *TGF-β1*-509C/T and 869T/C polymorphisms, 114 glioma patients and 138 cancer-free control individuals were analyzed. The statistical analysis of age distribution between control and glioma cases showed significant differences ($p \leq 0.001$). Regarding sex distribution, no significant differences were found between controls and cases ($p = 0.195$). The genotype and allele frequencies of the *TGF-β1*-509C/T and 869T/C polymorphisms in controls and glioma cases are shown in Table 2. The frequencies of the CC,

Table 3 Multivariate logistic regression analysis of the association between -509C/T and 869T/C polymorphisms and risk for each glioma group

Polymorphism	Control	Glioma (WHO grade 2–4)	OR (95 % CI) ^a	Glioblastoma (WHO grade 4)	OR (95 % CI) ^a
<i>TGF-β1</i> -509C/T					
Genotypes					
TT	22	17	–	15	–
CC	54	40	1.14 (0.45–2.98)	27	0.76 (0.27–2.13)
CT	62	52	1.11 (0.45–2.75)	39	0.82 (0.30–2.21)
CC+CT	116	92	1.13 (0.48–2.63)	66	0.79 (0.31–2.01)
Alleles					
T	0.384	0.394	–	0.426	–
C	0.616	0.606	1.08 (0.68–1.70)	0.574	0.89 (0.53–1.48)
Age			1.12 (1.09–1.15)		1.14 (1.10–1.18)
Sex					
Male	76	69	–	58	–
Female	62	40	0.40 (0.20–0.77)	27	0.24 (0.11–0.52)
<i>TGF-β1</i> 869T/C					
Genotypes					
CC	26	17	–	15	–
TT	48	40	1.36 (0.55–3.34)	29	0.89 (0.33–2.41)
TC	64	52	1.12 (0.47–2.66)	37	0.80 (0.31–2.07)
TT+TC	112	92	1.22 (0.55–2.73)	66	0.84 (0.35–2.02)
Alleles					
C	0.420	0.394	–	0.414	–
T	0.580	0.606	1.19 (0.75–1.87)	0.586	1.09 (0.64–1.84)
Age			1.12 (1.09–1.15)		1.14 (1.10–1.18)
Sex					
Male	76	69	–	58	–
Female	62	40	0.39 (0.20–0.76)	27	0.24 (0.11–0.52)

^a Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Bold-faced values indicate significant differences at 5 % level

CT, and TT genotypes of -509C/T were 39.1, 44.9, and 16.0 % in cancer-free controls, and 36.8, 47.4, and 15.8 % in glioma patients, respectively. Regarding the 869T/C polymorphism, the frequencies of the TT, TC, and CC genotypes were 34.8, 46.4, and 18.8 % in controls, and 36.8, 46.5, and 16.7 % in glioma cases, respectively. The distribution of -509C/T and 869T/C allele frequencies in the control group were in Hardy-Weinberg equilibrium ($p=0.891$ and $p=0.685$, respectively).

When assessing the allele frequencies of the *TGF-β1*-509C/T polymorphism by univariate analysis, we found that the C allele was not significantly associated with a higher risk for glioma (OR, 0.97; 95 % CI, 0.68–1.39; Table 2). Additionally, using TT genotype as reference, the OR analysis showed that the CC, CT, and combined CC+CT genotypes were not significantly associated with increased risk for glioma (OR, 0.95; 95 % CI, 0.45–1.98 for CC; OR, 1.07; 95 % CI, 0.52–2.19 for CT; OR, 1.01; 95 % CI, 0.51–2.00 for CC+CT; Table 2). Evaluating the *TGF-β1* 869T/C polymorphism by univariate analysis, the T allele was not significantly associated with a higher risk for glioma (OR, 1.09; 95 % CI, 0.76–1.56; Table 2). Using CC genotype as reference, the OR analysis showed that the TT, TC, and combined TT+TC genotypes were not significantly associated with increased risk for glioma (OR, 1.20; 95 % CI, 0.58–2.47 for TT; OR, 1.13; 95 % CI, 0.57–2.27 for TC; OR, 1.16; 95 % CI, 0.61–2.23 for TT+TC; Table 2). Taking into account that GBM were the most frequent subtype in our series ($n=85$), we also compared the control group with GBM cases. Using similar analysis, a lack of association between both *TGF-β1*-509C/T and 869T/C allele or genotype variants and risk for developing GBM was observed (Table 2). Moreover, for both polymorphisms, a multivariate logistic regression model adjusted for sex and age as a continuous variable (Table 3) was applied. As expected, increased age was associated with increased risks for developing glioma and GBM. Similarly, female gender was associated with decreased risks (Table 3). Consistent with the results observed by the univariate analysis, no associations between each polymorphic variant and risk for developing gliomas or GBMs were found (Table 3).

We then evaluated whether these *TGF-β1* polymorphisms may have an impact in patients' survival. To do so, we focused exclusively in GBM patients with available survival data ($n=44$), as glioma grade is a strong influencer of survival, precluding an analysis in the whole glioma dataset. Regarding -509C/T polymorphism, the Cox model showed that GBM patients carrying the TT genotype had significantly increased overall survival compared to those with the CC+CT genotypes (OR, 2.41; 95 % CI, 1.06–5.50; Table 4; $p=0.036$, Fig. 1a). Moreover, patients with CT genotype alone presented a shorter overall survival when compared to those carrying TT genotype (OR, 2.72; 95 % CI, 1.12–6.65; Table 4; $p=0.028$, Fig. 1b). No significant differences in overall survival

were found in GBM patients with *TGF-β1*-509CC versus TT genotypes (Table 4). Concerning the survival analysis for the *TGF-β1* 869T/C polymorphism, the Cox regression model demonstrated that TT+TC genotypes were significantly associated with shorter survival in GBM patients, as compared to the CC genotype (OR, 2.62; 95 % CI, 1.11–6.17; Table 4; $p=0.027$, Fig. 1c). These results were further supported when we compared patients with TC genotype with patients carrying the CC genotype (OR, 2.71; 95 % CI, 1.12–6.54; Table 4; $p=0.027$, Fig. 1d). No significant differences in overall survival were found in GBM patients with *TGF-β1* 869CC versus TT genotypes (Table 4).

Discussion

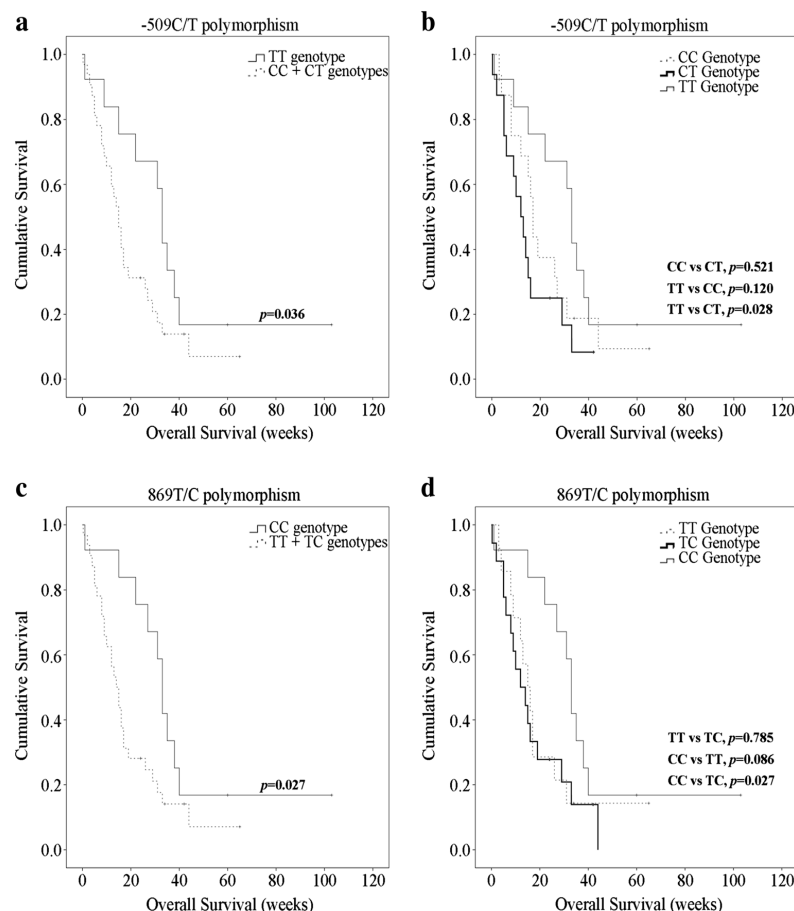
Gliomagenesis is a complex and poorly understood process in which genetic and environmental factors play critical roles. Several studies have suggested that SNPs are the most common sources of human genetic variation, and they may contribute to individual's susceptibility to cancer, including glioma [37]. So far, SNPs of several genes have been studied and identified as putative biomarkers for glioma susceptibility. Some examples include genes encoding proteins involved in

Table 4 Multivariate COX regression analysis of the association between -509C/T and 869T/C polymorphisms and survival in grade 4 gliomas

Polymorphism	Number of cases	OR (95 % CI) ^a
<i>TGF-β1</i> -509C/T		
Genotypes		
TT	12	–
CC	16	2.09 (0.83–5.31)
CT	16	2.72 (1.12–6.65)
CC+CT	32	2.41 (1.06–5.50)
Age		1.02 (0.99–1.05)
Sex		
Male	31	–
Female	13	1.13 (0.52–2.45)
<i>TGF-β1</i> 869T/C		
Genotypes		
CC	12	–
TT	14	2.43 (0.88–6.70)
TC	18	2.71 (1.12–6.54)
TT+TC	32	2.62 (1.11–6.17)
Age		1.01 (0.99–1.04)
Sex		
Male	31	–
Female	13	1.28 (0.57–2.89)

^a Odds ratio (OR) with 95 % confidence intervals (CI), adjusted for age (as a continuous variable) and sex. Bold-faced values indicate significant differences at 5 % level

Fig. 1 Effect of *TGF-β1*-509C/T and 869T/C polymorphisms in the survival of glioblastoma patients. Kaplan-Meier overall survival curves for *TGF-β1*-509C/T (a, b) and 869T/C (c, d) polymorphisms. In the -509C/T polymorphism, Cox regression analysis showed that the group of glioblastoma patients harboring CC+CT genotypes (a, $p=0.036$) or patients with CT genotype (b, $p=0.028$) had statistically significant shorter overall survivals when compared to patients with TT genotype. Regarding the 869T/C polymorphism, the group of glioblastoma patients with TT+TC genotypes (c, $p=0.027$) or patients with TC genotype (d, $p=0.027$) had significantly shorter overall survivals than patients with CC genotype. Tick marks indicate censored data



DNA repair pathways (*MGMT*, *PRKDC*, *ERCC1*, *XRCC1*, *APEX1*, *TP53*, *PARP1*, and *LIG1*) [38–41], cancer metabolism (*GST*, *CYP2D6*, *SOD2*, *SOD3*, *GPX1*, and *NOS1*) [42, 43], growth pathways [44, 45], among others [46, 47]. Many association studies on the *TGF-β1* polymorphisms have been conducted in several types of cancer, including lung [27], prostate [20, 26], gastric [21], hepatocellular [22], and breast cancers [19, 24, 30, 48]. To the best of our knowledge, this is the first study to evaluate the *TGF-β1*-509C/T and 869T/C polymorphisms in glioma patients. This is particularly relevant as these two polymorphisms have been reported to affect *TGF-β1* protein expression and influence the structure and function of *TGF-β1* peptide which may contribute to cancer [31, 32].

Using both univariate and multivariate statistical analyses, our results showed that none of the *TGF-β1*-509C/T and 869T/C polymorphisms are significantly associated with glioma susceptibility. These data fit well with previous studies in other tumor types in which 869T/C was not associated with breast cancer risk [48], and -509C/T polymorphism was not

associated with an increased risk of colorectal cancer [49]. While in our dataset, we included solely patients of Caucasian background, future studies should evaluate how these *TGF-β1* polymorphisms may have relevance in other ethnic backgrounds, as previously suggested for many other polymorphisms [50–53].

It has been described that *TGF-β1* contributes to cell growth, angiogenesis, and invasion, is highly active, and confers poor prognosis in high-grade glioma patients [16–18]. Therefore, it is conceivable that patients carrying the T allele of the -509C/T polymorphism and patients with the C allele of the 869T/C polymorphism may have reduced cancer survival, since both these alleles are associated with an elevated *TGF-β1* levels. Contrarily, in our study, GBM patients carrying TT genotype of the -509C/T polymorphism and patients with CC genotype of 869T/C polymorphism presented longer overall survival. This is in agreement with a previous work where it has been shown that breast cancer patients carrying the CC genotype of the 869T/C polymorphism presented a longer overall survival [24]. Therefore, the TT genotype of

the -509C/T polymorphism and the CC genotype of the 869T/C polymorphism have the potential to be used as predictive marker of better survival in patients with GBM. Additionally, taking into account that -509T allele has been suspected to increase the transcription of *TGF- β 1*, patients that present this variant may be more suited for an anti-TGF- β 1 monoclonal antibody therapy (Metelimumab) [54]. It remains, however, to be seen if -509C/T and 869T/C polymorphisms are in a linkage disequilibrium and if it is functionally relevant. For instance, it has been shown that these two *TGF- β 1* polymorphisms (-509C/T and 869T/C) are in strong linkage disequilibrium in breast cancer patients, although it remains to be determined which of the two polymorphisms is functionally significant and affect survival [25].

In conclusion, this study shows that *TGF- β 1*-509C/T and 869T/C polymorphisms do not confer susceptibility to develop glioma but may have an impact in the survival of GBM patients. Specifically, the *TGF- β 1*-509TT and 869CC genotypes can be used as predictive markers of improved survival. In the future, additional studies with larger datasets will be needed to extend and validate these novel findings.

Acknowledgments This work was supported by Fundação para a Ciência e Tecnologia, Portugal (PTDC/SAU-GMG/113795/2009 and SFRH/BPD/33612/2009 to B.M.C.; SFRH/BD/88121/2012 to J.V.C.; SFRH/BD/92786/2013 to C.S.G.; PTDC/SAU-ONC/115513/2009 to R.M.R.).

Conflict of interest None.

References

- Boyle P, Levin B. World cancer report 2008. IARC Press, International Agency for Research on Cancer, 2008.
- Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol*. 2006;2:494–503. quiz 491 p following 516.
- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 who classification of tumours of the central nervous system. *Acta Neuropathol*. 2007;114:97–109.
- Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807–12.
- Butowski NA, Sneed PK, Chang SM. Diagnosis and treatment of recurrent high-grade astrocytoma. *J Clin Oncol*. 2006;24:1273–80.
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987–96.
- Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, et al. Brain tumor epidemiology: consensus from the brain tumor epidemiology consortium. *Cancer*. 2008;113:1953–68.
- Reuss D, von Deimling A. Hereditary tumor syndromes and gliomas. *Recent Results Cancer Res*. 2009;171:83–102.
- Wrensch M, Lee M, Miike R, Newman B, Barger G, Davis R, et al. Familial and personal medical history of cancer and nervous system conditions among adults with glioma and controls. *Am J Epidemiol*. 1997;145:581–93.
- Hocking B. Occupational exposure to ionizing and non-ionizing radiation and risk of glioma. *Occup Med (Lond)*. 2008;58:148–9. author reply 149.
- Ron E, Modan B, Boice Jr JD, Alfandary E, Stovall M, Chetrit A, et al. Tumors of the brain and nervous system after radiotherapy in childhood. *N Engl J Med*. 1988;319:1033–9.
- Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B, et al. Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet*. 2009;41:899–904.
- Wrensch M, Jenkins RB, Chang JS, Yeh RF, Xiao Y, Decker PA, et al. Variants in the *cdkn2b* and *rtel1* regions are associated with high-grade glioma susceptibility. *Nat Genet*. 2009;41:905–8.
- Massague J. Tgfbeta in cancer. *Cell*. 2008;134:215–30.
- Derynck R, Zhang YE. Smad-dependent and smad-independent pathways in tgf-beta family signalling. *Nature*. 2003;425:577–84.
- Kaminska B, Kocyk M, Kijewska M. Tgf beta signaling and its role in glioma pathogenesis. *Adv Exp Med Biol*. 2013;986:171–87.
- Rich JN. The role of transforming growth factor-beta in primary brain tumors. *Front Biosci*. 2003;8:e245–60.
- Penuelas S, Anido J, Prieto-Sanchez RM, Folch G, Barba I, Cuatras I, et al. Tgf-beta increases glioma-initiating cell self-renewal through the induction of *l1f* in human glioblastoma. *Cancer Cell*. 2009;15:315–27.
- Dunning AM, Ellis PD, McBride S, Kirschenlohr HL, Healey CS, Kemp PR, et al. A transforming growth factorbeta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. *Cancer Res*. 2003;63:2610–5.
- Cai Q, Tang Y, Zhang M, Shang Z, Li G, Tian J, et al. Tgfbeta1 leu10pro polymorphism contributes to the development of prostate cancer: evidence from a meta-analysis. *Tumour Biol*. 2014;35:667–73.
- Chang WW, Zhang L, Su H, Yao YS. An updated meta-analysis of transforming growth factor-beta1 gene: three polymorphisms with gastric cancer. *Tumour Biol*. 2014;35:2837–44.
- Migita K, Miyazoe S, Maeda Y, Daikoku M, Abiru S, Ueki T, et al. Cytokine gene polymorphisms in japanese patients with hepatitis b virus infection—association between tgf-beta1 polymorphisms and hepatocellular carcinoma. *J Hepatol*. 2005;42:505–10.
- Li K, Xia F, Zhang K, Mo A, Liu L. Association of a tgf-beta1-509c/t polymorphism with gastric cancer risk: a meta-analysis. *Ann Hum Genet*. 2013;77:1–8.
- Gonzalez-Zuloeta Ladd AM, Arias-Vasquez A, Siemes C, Coebergh JW, Hofman A, Witterman J, et al. Transforming-growth factor beta1 leu10pro polymorphism and breast cancer morbidity. *Eur J Cancer*. 2007;43:371–4.
- Shu XO, Gao YT, Cai Q, Pierce L, Cai H, Ruan ZX, et al. Genetic polymorphisms in the tgf-beta 1 gene and breast cancer survival: a report from the shanghai breast cancer study. *Cancer Res*. 2004;64:836–9.
- Ewart-Toland A, Chan JM, Yuan J, Balmain A, Ma J. A gain of function tgfbeta1 polymorphism may be associated with late stage prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2004;13:759–64.
- Kang HG, Chae MH, Park JM, Kim EJ, Park JH, Kam S, et al. Polymorphisms in tgf-beta1 gene and the risk of lung cancer. *Lung Cancer*. 2006;52:1–7.
- Luedecking EK, DeKosky ST, Mehdi H, Ganguli M, Kamboh MI. Analysis of genetic polymorphisms in the transforming growth factor-beta1 gene and the risk of alzheimer's disease. *Hum Genet*. 2000;106:565–9.
- Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, et al. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum Mol Genet*. 1999;8:93–7.
- Kaklamani VG, Baddi L, Liu J, Rosman D, Phukan S, Bradley C, et al. Combined genetic assessment of transforming growth factor-

- beta signaling pathway variants may predict breast cancer risk. *Cancer Res.* 2005;65:3454–61.
31. Watanabe Y, Kinoshita A, Yamada T, Ohta T, Kishino T, Matsumoto N, et al. A catalog of 106 single-nucleotide polymorphisms (snps) and 11 other types of variations in genes for transforming growth factor-beta1 (tgf-beta1) and its signaling pathway. *J Hum Genet.* 2002;47:478–83.
 32. Li X, Yue ZC, Zhang YY, Bai J, Meng XN, Geng JS, et al. Elevated serum level and gene polymorphisms of tgf-beta1 in gastric cancer. *J Clin Lab Anal.* 2008;22:164–71.
 33. Blobel GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med.* 2000;342:1350–8.
 34. Elliott RL, Blobel GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol.* 2005;23:2078–93.
 35. Derynck R, Akhurst RJ, Balmain A. Tgf-beta signaling in tumor suppression and cancer progression. *Nat Genet.* 2001;29:117–29.
 36. Mullenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends Genet.* 1989;5:391.
 37. Wu GY, Hasenberg T, Magdeburg R, Bonninghoff R, Sturm JW, Keese M. Association between egf, tgf-beta1, vegf gene polymorphism and colorectal cancer. *World J Surg.* 2009;33:124–9.
 38. Lima-Ramos V, Pacheco-Figueiredo L, Costa S, Pardo F, Silva A, Amorim J, et al. Tp53 codon 72 polymorphism in susceptibility, overall survival, and adjuvant therapy response of gliomas. *Cancer Genet Cytogenet.* 2008;180:14–9.
 39. Liu Y, Scheurer ME, El-Zein R, Cao Y, Do KA, Gilbert M, et al. Association and interactions between DNA repair gene polymorphisms and adult glioma. *Cancer Epidemiol Biomarkers Prev.* 2009;18:204–14.
 40. Wang LE, Bondy ML, Shen H, El-Zein R, Aldape K, Cao Y, et al. Polymorphisms of DNA repair genes and risk of glioma. *Cancer Res.* 2004;64:5560–3.
 41. Wiencke JK, Aldape K, McMillan A, Wiemels J, Moghadassi M, Miike R, et al. Molecular features of adult glioma associated with patient race/ethnicity, age, and a polymorphism in o6-methylguanine-DNA-methyltransferase. *Cancer Epidemiol Biomark Prev.* 2005;14:1774–83.
 42. Elexpuru-Camirua J, Buxton N, Kandula V, Dias PS, Campbell D, McIntosh J, et al. Susceptibility to astrocytoma and meningioma: influence of allelism at glutathione s-transferase (gstt1 and gstm1) and cytochrome p-450 (cyp2d6) loci. *Cancer Res.* 1995;55:4237–9.
 43. Zhao P, Zhao L, Zou P, Lu A, Liu N, Yan W, et al. Genetic oxidative stress variants and glioma risk in a chinese population: a hospital-based case-control study. *BMC Cancer.* 2012;12:617.
 44. Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A, et al. Association between functional egf+61 polymorphism and glioma risk. *Clin Cancer Res.* 2007;13:2621–6.
 45. Costa BM, Viana-Pereira M, Fernandes R, Costa S, Linhares P, Vaz R, et al. Impact of egfr genetic variants on glioma risk and patient outcome. *Cancer Epidemiol Biomarkers Prev.* 2011;20:2610–7.
 46. Jiang H, Lian M, Xie J, Li J, Wang M. Three single nucleotide polymorphisms of the vascular endothelial growth factor (vegfr) gene and glioma risk in a chinese population. *J Int Med Res.* 2013;41:1484–94.
 47. Pandey JP, Kaur N, Costa S, Amorim J, Nabico R, Linhares P, et al. Immunoglobulin genes implicated in glioma risk. *Oncoimmunology.* 2014;3:e28609.
 48. Krippel P, Langsenlehner U, Renner W, Yazdani-Biuki B, Wolf G, Wascher TC, et al. The 110p polymorphism of the transforming growth factor-beta 1 gene is not associated with breast cancer risk. *Cancer Lett.* 2003;201:181–4.
 49. Qi P, Ruan CP, Wang H, Zhou FG, Zhao YP, Gu X, et al. 509c>t polymorphism in the tgf-beta1 gene promoter is not associated with susceptibility to and progression of colorectal cancer in chinese. *Color Dis: Off J Assoc Coloproctol G B Irel.* 2010;12:1153–8.
 50. Crivello A, Giacalone A, Vaglica M, Scola L, Forte GI, Macaluso MC, et al. Regulatory cytokine gene polymorphisms and risk of colorectal carcinoma. *Ann N Y Acad Sci.* 2006;1089:98–103.
 51. Macarthur M, Sharp L, Hold GL, Little J, El-Omar EM. The role of cytokine gene polymorphisms in colorectal cancer and their interaction with aspirin use in the northeast of scotland. *Cancer Epidemiol Biomarkers Prev.* 2005;14:1613–8.
 52. Saltzman BS, Yamamoto JF, Decker R, Yokochi L, Theriault AG, Vogt TM, et al. Association of genetic variation in the transforming growth factor beta-1 gene with serum levels and risk of colorectal neoplasia. *Cancer Res.* 2008;68:1236–44.
 53. Zhang Y, Liu B, Jin M, Ni Q, Liang X, Ma X, et al. Genetic polymorphisms of transforming growth factor-beta1 and its receptors and colorectal cancer susceptibility: a population-based case-control study in china. *Cancer Lett.* 2009;275:102–8.
 54. Benigni A, Zoja C, Corna D, Zatelli C, Conti S, Campana M, et al. Add-on anti-tgf-beta antibody to ace inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol: JASN.* 2003;14:1816–24.

5 INFLUENCE OF NEUTROPHIL -LYMPHOCYTE RATIO IN PROGNOSIS OF GLIOBLASTOMA MULTIFORME

Marta Lopes,¹ Bruno Carvalho,^{2,3} Rui Vaz,^{2,3,4} Paulo Linhares^{2,3,4}

¹ Department of Neurology, Hospital São Sebastião, Entre Douro e Vouga Hospital Centre, Rua Dr. Cândido de Pinho, 4520-211 Santa Maria da Feira, Portugal; ² Department of Neurosurgery, Hospital São João, São João, Hospital Centre, Oporto, Portugal; ³ Faculty of Medicine, Oporto University, Oporto, Portugal; ⁴ Neurosciences Center, Hospital CUF Porto, Oporto, Portugal.

Influence of neutrophil–lymphocyte ratio in prognosis of glioblastoma multiforme

Marta Lopes¹  · Bruno Carvalho^{2,3} · Rui Vaz^{2,3,4} · Paulo Linhares^{2,3,4}

Received: 13 December 2016 / Accepted: 22 October 2017
© Springer Science+Business Media, LLC 2017

Abstract Neutrophil–lymphocyte ratio (NLR) is a hematological marker of systemic inflammation and several studies demonstrate an association between a higher NLR and a worse prognosis in many malignancies. However, literature analyzing its prognostic value in glioblastoma multiforme (GBM) is still scarce. We intended to analyze the correlation of NLR with overall survival and progression-free survival in patients with GBM performing a retrospective review of the patients with diagnosis of GBM submitted to a resection surgery in the department of neurosurgery of a tertiary care hospital, between January/2005 and January/2013. 140 patients were included. Mean age at surgery was 62.9 ± 10.0 years and mean age at death was 64.4 ± 9.8 years. Mean overall survival was 19.4 ± 14.3 months and mean progression-free survival was 9.4 ± 8.7 months. There was no correlation of NLR, platelets-lymphocyte ratio (PLR) or absolute counts of neutrophils, lymphocytes and platelets with overall survival in multivariate analysis. However, a preoperative $NLR \leq 5$ correlated with a shorter progression-free survival [HR 1.56 (SD 95% 1.04–2.34); $p=0.032$]. We performed a subgroup analysis of patients who completed Stupp protocol. In this subgroup of 117 patients, a preoperative $NLR > 7$ correlated with a shorter overall survival [HR 1.65 (SD 95% 1.07–2.53); $p=0.023$]. The results from our

total cohort didn't confirm the correlation between a higher NLR and worse survival in GBM. However, in the subgroup analysis of patients who completed Stupp protocol, a higher NLR was an independent prognostic factor to a shorter overall survival, similar to existent literature data about GBM.

Keywords Glioblastoma multiforme · Neutrophil–lymphocyte ratio · Epidemiology · Overall survival · Progression-free survival

Introduction

Cancer-associated inflammation promotes angiogenesis and proliferation of malignant cells, thereby favoring disease progression [1]. NLR is a hematological marker of systemic inflammation and has been pointed as an independent risk factor for worse prognosis in several malignancies [2–4].

GBM remains a poor prognosis malignancy and in the temozolomide era survival rate 2 years after diagnosis remains about 26.5% [5].

There are several well studied prognostic factors in GBM, such as age at diagnosis, preoperative KPS and MMSE, tumour location and volume, extent of tumour resection, type of systemic therapy, histologic findings and molecular genetic factors [6–8]. A deeper knowledge about prognostic factors in GBM allows guiding the most appropriate adjuvant treatment in a case-by-case basis.

A few studies analyzed the prognostic value of NLR in GBM patients and higher values were found to be an independent poor prognostic factor. However, literature under this subject is still scarce. There are only 4 completed retrospective studies with total populations of 84 [9], 107 [10], 152 [11] and 166 [12] patients, and preliminary results from one prospective study [13].

✉ Marta Lopes
marta.fmup@gmail.com

¹ Department of Neurology, Hospital São Sebastião, Entre Douro e Vouga Hospital Centre, Rua Dr. Cândido de Pinho, 4520-211 Santa Maria da Feira, Portugal

² Department of Neurosurgery, Hospital São João, São João Hospital Centre, Oporto, Portugal

³ Faculty of Medicine, Oporto University, Oporto, Portugal

⁴ Neurosciences Center, Hospital CUF Porto, Oporto, Portugal

Platelets-lymphocyte ratio (PLR) is another marker of host inflammation but its prognostic role in GBM is less studied than NLR. Only 2 studies analyzed this marker in GBM. One found PLR to be an independent prognostic factor, with low PLR being associated with better prognosis [12]. In the other study despite significant in univariate analysis, PLR was not an independent prognostic factor in multivariate analysis [11].

At the present study, we intended to analyze the influence of NLR in overall survival and progression-free survival in patients with GBM. We also evaluated the prognostic value of PLR and absolute counts of neutrophils, lymphocytes and platelets. We hypothesized that higher pretreatment NLR and PLR are independent poor prognostic factors in GBM patients submitted to tumour resection surgery.

Methods

Study design and setting

This is a retrospective patient cohort study of patients with GBM who were submitted to a resection surgery in the neurosurgery unit of a tertiary care hospital.

Clinical data collection

We retrospectively reviewed a prospectively maintained database of patients with cerebral tumours and selected the patients with GBM who were submitted to a resection surgery between January 2005 and January 2013. We also reviewed the clinical registries of all patients. We collected all demographic and clinical data of the included patients from the database and clinical registries. We excluded patients with no clinical information in these sources. Regrowth tumours were also excluded. We collected neutrophil, lymphocyte and platelet values from the full blood counts performed before surgery, in the 24 h post-surgery and in the first appointment before the beginning of adjuvant chemoradiotherapy.

Diagnostic criteria

We considered overall survival as the time frame between the first resection surgery and death. In patients with unknown date of death we considered the date of the last follow-up appointment. We considered progression-free survival as the time frame between surgery and the date of tumour progression documentation in brain magnetic resonance imaging. NRL was calculated as absolute neutrophil count divided by absolute lymphocyte count. PLR was calculated as absolute platelet count divided by absolute lymphocyte count.

Data analysis

We performed univariate and multivariate analysis using the Cox proportional hazard method to ascertain the influence of variables on overall survival and progression-free survival. We built a multivariate linear regression model using the stepwise backward likelihood ratio method. We tested as potential confounders: sex, age at surgery, tumour location (predominantly frontal, temporal, parietal, occipital or other locations), extent (1 lobe, 2 lobes or more than 2 lobes) and focality (uni or multifocal), involvement of eloquent areas, comorbidities with potential impact in NLR (hypertension, diabetes mellitus, metabolic syndrome, cardiac, hepatic or renal disease and inflammatory disorders), first-line adjuvant systemic therapy, second-line adjuvant systemic therapy, surgery resection extent and preoperative KPS. NLR was analysed as a continuous and dichotomous variable. Based on previous studies, in order to find which NLR cut-off had the most significant associated p-value, we tested 7 successive cut-off values between 2.0 and 8.0 using the multivariate model. To analyse the differences between groups we used Person Qui-square, Mann–Whitney U test or Kruskal–Wallis test as appropriate. We also performed a subgroup survival analysis using the same methods described above in the subgroup of patients who completed Stupp protocol. We considered as statistically significant a p value <0.05. The software used for statistical analysis was IBM SPSS Statistics 22.

Results

We included 140 patients. Demographic and clinical characteristics of our cohort are detailed in Table 1. Male-to-female ratio was 2.3:1. Mean age at surgery was 62.9 ± 10.0 years and mean age at death was 64.4 ± 9.8 years. Mean overall survival was 19.4 ± 14.3 months and mean progression-free survival was 9.4 ± 8.7 months.

In multivariate linear regression analysis, we found that occipital tumour location, absence of first-line systemic therapy, absence of second-line systemic therapy, presence of comorbidities and a lower preoperative KPS were correlated with a shorter overall survival (Table 2). Absence of first-line systemic therapy, absence of second-line systemic therapy and tumour multifocality were correlated with a shorter progression-free survival (Table 3).

Measured as continuous variables, there was no correlation of NLR, PLR and absolute number of neutrophils, lymphocytes and platelets, from full blood counts preoperative, in the 24-h post-surgery and in the first appointment before chemoradiotherapy, with overall survival or progression-free survival. There was also no correlation of preoperative NLR, measured as a categorical variable, with overall survival, but

Table 1 Demographic and clinical characteristics of the included patients

Characteristics	N	Mean $\pm$ SD or percentage
Gender		
Female	42	30.0%
Male	98	70.0%
Age at surgery (years)	140	62.9 $\pm$ 10.0
Age at death (years)	140	64.5 $\pm$ 9.7
Overall survival (months)	140	19.4 $\pm$ 14.3
Progression-free time (months)	132	9.4 $\pm$ 8.7
Tumor location		
Temporal	41	29.3%
Frontal	34	24.3%
Other	65	46.4%
Tumor extension		
1 lobe	88	62.9%
2 lobes	46	32.9%
> 2 lobes	6	4.3%
Tumor focality		
Unifocal	121	86.4%
Multifocal	19	13.6%
Eloquent area involvement		
Yes	63	45.3%
No	76	54.7%
Comorbidities with potential impact in NLR		
Yes	74	55.2%
No	60	44.8%
First-line adjuvant systemic therapy		
STUPP	117	83.6%
Other	9	6.4%
None	14	10.0%
Second-line adjuvant systemic therapy		
Yes	64	45.7%
No	76	54.3%
Surgery resection extent		
Partial	38	27.1%
Subtotal	44	31.4%
Total	58	41.4%
Preoperative KPS	129	82.6 $\pm$ 14.2
Preoperative NLR	126	9.0 $\pm$ 6.5
Preoperative PLR	126	218.8 $\pm$ 122.1

a preoperative NLR ≤ 5 correlated with a shorter progression-free survival [$p=0.032$; HR 1.56 (1.04–2.34)] (Fig. 1).

We also evaluated the difference between postoperative and preoperative counts of NLR, PLR, neutrophil, lymphocyte and platelet, but none of these variables correlated with overall survival or progression-free survival.

The groups of patients with NLR ≤ 5 and > 5 are statistically comparable, except in the type of first-line systemic therapy ($p=0.001$). The patients submitted to Stupp protocol tend to have NLR > 5 while patients submitted to other therapeutic schemas tend to have NLR ≤ 5 . All other

demographic and clinical characteristics are similar between groups namely gender ($p=0.206$), age at surgery ($p=0.609$), tumour extension ($p=0.789$), location ($p=0.739$) and focality ($p=0.360$), involvement of eloquent areas ($p=0.981$), comorbidities ($p=0.324$), second-line systemic therapy ($p=0.431$), surgery resection extent ($p=0.838$) and preoperative KPS ($p=0.464$).

Stupp protocol was completed in 117 patients. In 14 patients, any adjuvant treatment was made and 9 patients received other treatment modalities (only standard or hypofractionated radiotherapy, only chemotherapy with

Table 2 Univariate and multivariate linear regression analysis of variables influence on overall survival

Variable	Univariate analysis		Multivariate analysis [†]	
	P value	HR [95% CI]	P value	HR [95% CI]
Sex	0.919	1.02 [0.71–1.47]	–	–
Age at surgery	0.005*	1.02 [1.01–1.04]	–	–
Tumor location	0.143	1.05 [0.99–1.11]	0.014*	1.08 [1.02–1.16]
Tumor extent	0.294	1.17 [0.87–1.59]	–	–
Tumor focality	0.007*	1.97 [1.20–3.25]	–	–
Involvement of eloquent areas	0.014*	1.54 [1.09–2.17]	–	–
Comorbidities	0.022*	1.50 [1.06–2.11]	0.044*	1.47 [1.01–2.13]
First-line systemic therapy	0.486	0.80 [0.44–1.49]	0.020*	0.38 [0.17–0.86]
Second-line systemic therapy	0.040*	0.70 [0.50–0.98]	0.034*	0.67 [0.47–0.97]
Surgery resection extent	0.468	0.93 [0.76–1.14]	–	–
Preoperative KPS	0.001*	0.98 [0.97–0.99]	0.000*	0.98 [0.96–0.99]
Preoperative NLR	0.395	1.01 [0.99–1.04]	0.868	1.00 [0.97–1.03]
24 h post-surgery NLR	0.976	1.00 [0.98–1.02]	0.820	1.00 [0.98–1.02]
First appointment post-surgery NLR	0.297	1.03 [0.98–1.07]	0.520	1.02 [0.97–1.07]
Preoperative PLR	0.878	1.00 [1.00–1.00]	0.951	1.00 [1.00–1.00]
Preoperative neutrophils	0.340	1.03 [0.98–1.08]	0.728	1.01 [0.96–1.07]
Preoperative lymphocytes	0.960	0.99 [0.790–1.25]	0.668	0.95 [0.74–1.21]
Preoperative platelets	0.233	1.00 [1.00–1.00]	0.585	1.00 [1.00–1.00]

KPS Karnofsky performance score, NLR neutrophil–lymphocyte ratio, PLR platelet–lymphocyte ratio

[†]Using the stepwise backward likelihood ratio method

*Statistically significant (p < 0.05)

Table 3 Univariate and multivariate linear regression analysis of variables influence on progression-free survival

Variable	Univariate analysis		Multivariate analysis [†]	
	P value	HR [95% CI]	P value	HR [95% CI]
Sex	0.690	0.93 [0.63–1.36]	–	–
Age at surgery	0.009*	1.02 [1.01–1.04]	–	–
Tumor location	0.531	1.02 [0.96–1.08]	–	–
Tumor extent	0.964	0.99 [0.71–1.39]	–	–
Tumor focality	0.005*	2.09 [1.25–3.50]	0.004*	2.11 [1.27–3.53]
Involvement of eloquent areas	0.417	1.16 [0.82–1.64]	–	–
Comorbidities	0.010*	1.61 [1.12–2.31]	–	–
First-line systemic therapy	0.003*	0.28 [0.12–0.64]	0.002*	0.30 [0.14–0.65]
Second-line systemic therapy	0.040*	0.70 [0.49–0.98]	0.027*	0.68 [0.48–0.96]
Surgery resection extent	0.621	0.95 [0.77–1.17]	–	–
Preoperative KPS	0.050	0.99 [0.97–1.00]	–	–
Preoperative NLR (continuous)	0.662	1.01 [0.98–1.04]	0.653	0.99 [0.93–1.05]
Preoperative NLR (≤ 5.0 V s > 5.0)	0.165	1.31 [0.89–1.93]	0.032*	1.56 [1.04–2.34]
24 h post-surgery NLR	0.184	1.01 [1.00–1.03]	0.320	1.01 [0.99–1.03]
First appointment post-surgery NLR	0.221	1.03 [0.99–1.07]	0.328	1.02 [0.98–1.07]
Preoperative PLR	0.908	1.00 [1.00–1.00]	0.700	1.00 [1.00–1.00]
Preoperative neutrophils	0.603	0.99 [0.93–1.04]	0.653	0.99 [0.93–1.05]
Preoperative lymphocytes	0.694	0.95 [0.75–1.21]	0.961	1.01 [0.79–1.28]
Preoperative platelets	0.239	1.00 [1.00–1.00]	0.309	1.00 [1.00–1.00]

KPS Karnofsky performance score, NLR neutrophil–lymphocyte ratio, PLR platelet–lymphocyte ratio

[†]Using the stepwise backward likelihood ratio method

*Statistically significant (p < 0.05)

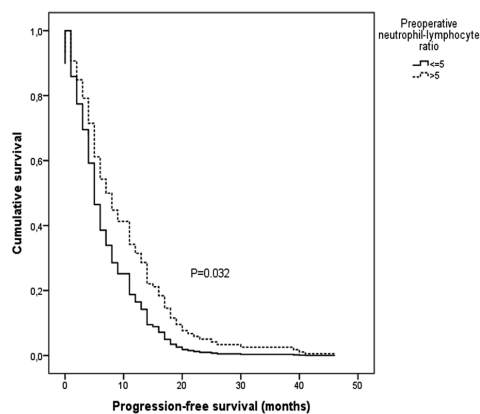


Fig. 1 Kaplan-Meier plot of progression-free survival stratified by neutrophil-lymphocyte ratio value (cut-off = 5.0)—whole study population

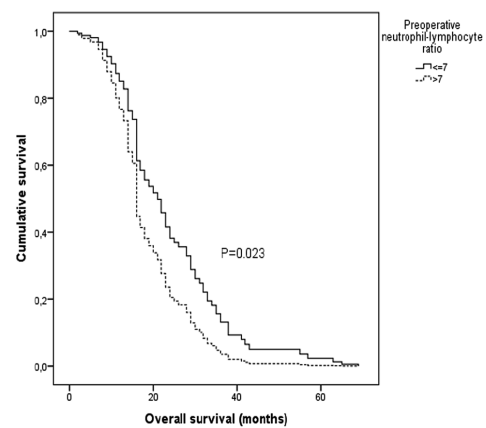


Fig. 2 Kaplan-Meier plot of overall survival stratified by neutrophil-lymphocyte ratio value (cut-off = 7.0)—Stupp-protocol treated patients

temozolomide or carmustine), due to poor functional status. We compared the 3 treatment groups and found a statistically significant difference in NLR, with none and other treatments groups having lower NRL values than Stupp protocol group (Stupp 9.48 ± 6.37 ; Other treatments: 3.16 ± 1.49 ; None: 9.04 ± 7.54 ; $p = 0.003$). Stupp patients are significantly younger than patients in other treatment groups (Stupp: 61.4 ± 10.0 ; Other treatments: 71.7 ± 4.2 ; None: 69.7 ± 6.1 ; $p = 0.000$) and had significantly more total surgical tumour resections ($p = 0.001$). In survival analysis, we found Stupp patients to have significantly longer overall survival (Stupp: 21.3 ± 12.7 ; other treatments: 11.2 ± 11.4 ; None: 9.4 ± 22.2) and progression-free survival (Stupp: 10.2 ± 8.4 ; Other treatments: 8.0 ± 14.3 ; None: 1.0 ± 1.3) than the remaining patients.

We performed a subgroup analysis of patients who completed Stupp protocol ($n = 117$). In multivariate linear regression analysis, higher age at diagnosis [$p = 0.005$; HR 1.03 (1.01–1.05)], tumour multifocality [$p = 0.003$; HR 2.34 (1.35–4.06)] and involvement of eloquent areas [$p = 0.008$; HR 1.68 (1.14–2.47)] correlated with a shorter overall survival, while tumour multifocality [$p = 0.001$; HR 2.42 (1.40–4.17)] predicted shorter progression-free survival.

A preoperative NLR > 7 correlated with a shorter overall survival [$p = 0.023$; HR 1.65 (1.07–2.53)] (Fig. 2). There was no correlation of preoperative NLR with progression-free survival.

Discussion

Estimating the pre-treatment prognosis is crucial to provide the most appropriate individual treatment [14]. Therefore,

the design of increasingly accurate prognostic scales is required [6, 15]. The molecular biomarkers are beginning to play an important role in determining the prognosis of brain tumours [16].

Mutations of IDH1 has been identified as prognostic markers for GBM [17]. Patients with IDH1 mutations had a significantly improved prognosis, with a median overall survival of 3.8 years as compared to 1.1 years for patients with wild-type IDH1 [18]. Also, Sanson et al. demonstrate improved progression free survival in their set of patients with GBM with 55 months PFS in patients with IDH1 mutation versus 8.8 months PFS in those without it [19]. IDH1 mutations are more frequently found in secondary glioblastomas, with reported frequencies ranging from 50 to 86% compared with primary glioblastomas, which contain the mutation only 4–21% of the time [20]. These secondary glioblastomas have itself a better prognosis than the primary ones. The meta-analysis published by Chen et al. [21] after reviewing a pool of 24 out 165 studies, concluded that IDH1/2 mutations are associated with improved survival in patients with glioblastomas. Several studies have found an association between TP53 and IDH1 mutations in astrocytic tumours [22, 23].

The 2016 update of the WHO classification of CNS tumours incorporates the molecular parameters, as a marker of prognosis and treatment of these entities [24]. Unfortunately, in our service only in 2016 it was possible to begin to search the mutations of IDH and MGMT. The absence of these data did not allow us to correlate them with the NLR.

The tumorigenesis process involves a complex interaction of cells in a particular microenvironment. Among these, inflammation seems to play an important role. Necrotic cell

death releases pro-inflammatory signs to this microenvironment and that immune inflammatory cells can be actively tumor promoting, providing angiogenesis, cell proliferation, and invasiveness. Tumor-associated inflammatory response has the paradoxical effect of enhancing tumorigenesis and progression, by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis [1]. The role of neutrophils in blood and tumor tissues was addressed in a review paper by Donskov [2]. A higher inflammation state correlates with worse prognosis in many neoplastic diseases [1, 2, 4, 25]. There is a correlation between glial tumour grade and the extension of neutrophils infiltration, with 85% of glioblastomas presenting a high infiltration compared to only 45–50% of the low-grade gliomas. They also find a significant positive correlation between circulating neutrophil count and extent of neutrophil infiltration into the tumor [26].

The prognostic value of NLR in peripheral blood, a marker of systemic inflammatory responses, was identified in glioblastoma and is a marker of higher neutrophil and lower T-cell infiltration in GBM [11, 27]. NLR is an easy obtainable value, from full blood count. However, it can be influenced by several factors, such as cardiovascular disease [28], hypertension [29], insulin resistance [30], uraemia [31], autoimmune disorders [32, 33] and glucocorticoid therapy [34]. In the present work, we adjusted the results to patient comorbidities that could potentially influence the inflammatory cell counts.

Several studies analysed the influence of preoperative NLR in prognosis of GBM. Bambury et al. [9] showed a significant correlation in univariate analysis between $\text{NLR} > 4$ and a worse median overall survival and in multivariate analysis $\text{NLR} > 4$ remained an independent prognostic indicator for poor outcome. Han et al. [11] showed $\text{NLR} \geq 4$ to be an independent predictor of worse overall survival. They also studied histological specimens for neutrophil and CD3+ infiltration and found that high pre-treatment NLR was significantly associated with high neutrophil infiltration and low CD3+ T-cell infiltration into tumours. Wang et al. [12], in a more recently published work studied the prognostic value of NLR considering the IDH mutation status. A $\text{NLR} \leq 4$ was found to be associated with better overall survival only in the group of GBM carrying IDH-1 wild-type, but not in GBM or primary GBM groups. Alexiou et al. [13] published preliminary results of the only prospective study and achieved a cut-off NLR value of 4.73 as best predicting survival. McNamara et al. [10], differently from the previous studies, included only recurrent GBM and found a median overall survival in patients with $\text{NLR} \leq 4$ versus $\text{NLR} > 4$ of 9.7 and 5.9 months, respectively.

In our work, contrasting with the previous studies, a higher NLR didn't correlate with a shorter overall survival and progression-free survival. In fact, as opposed to current literature, we found a $\text{NLR} \leq 5$ to be a predictor of shorter progression-free survival. This result is difficult to explain in light of the current knowledge, and it can be due to statistical bias, because the number of patients in other treatment groups is low. When we analysed the differences between treatment groups we found that patients submitted to Stupp protocol are younger, had more total tumour resections and longer overall survival and progression free survival. This are expected results because patients who are not selected for Stupp protocol have poorer functional status. However, interestingly, NLR is significantly higher in patients in Stupp protocol group. The difference is particularly striking when comparing with the group of patients submitted to other adjuvant protocols. This can be due solely to statistical bias because the number of patients is low in non-Stupp groups. Other hypothesis is that patients with very low survival categories have lower NLR, but larger and controlled studies are needed.

Because of the potential statistical bias derived from including patients submitted to other therapeutic schemes, we performed a subgroup analysis of the patients who completed the Stupp protocol treatment, the gold-standard adjuvant treatment in GBM. These patients have better performance scores (mean KPS = 83.9 vs. 75.3) and are submitted to the treatment with best prognosis, thus are the patients who benefit most of a detailed prognostic analysis. In this subgroup of patients, a $\text{NLR} > 7$ correlated with a shorter overall survival, and this result is more congruent with literature data.

We did not make any correlation with the MGMT status due to the technical incapacity to obtain this marker, but reviewing the literature seems there are no correlations establish between NLR and MGMT promoter methylation status, and the prognostic role of NLR was not significantly modified by the MGMT promoter methylation status [11]. We recognize, however, the high prognostic value of molecular markers tests and we consider that in future research its inclusion should be mandatory.

Another inflammatory marker, the PLR, was also investigated as a possible prognostic marker in glioblastomas. Han et al. demonstrates a significant correlation between PLR and patient survival in univariate analysis, but in multivariate analysis, the prognostic significance of PLR was markedly diminished [11]. In our study, there was no correlation between PLR and progression-free survival or overall survival. This is in accordance with the results of Han et al. that find a NLR superior to PLR as a prognostic factor in glioblastoma. However, in a more recent study of Wang et al. [12] PLR was found to be an independent prognostic factor, with low PLR being associated with better prognosis.

Our study has several limitations. Being a retrospective cohort study, it is subject to bias. The neurooncology inpatients protocol for newly admitted patients with cerebral tumours is fully implemented for several years. As part of the service protocol, blood samples shall be collected at patients' admission, before the administration of any treatment. Being so, despite we cannot confirm the timing of the first CCT dose given the retrospective and not controlled nature of the present study, we believe the protocol was accomplished. The prognostic value of other inflammatory markers, such as C-reactive protein and albumin, nor the value of a combined inflammation-based score, were not analysed as has been suggested [35–37]. Still, we gathered a relatively large cohort of patients.

Therapies are under investigation in order to reduce neutrophils and possibly improve prognosis [38]. However, a high baseline neutrophil count in either tumour or blood, or both, was identified as a strong, independent risk factor for poor outcome and the negative prognostic impact of neutrophils was not eliminated by increasing the dose of cytokines, chemotherapy, or targeted therapy [26].

In conclusion, in contrast with the results of previous published studies, we haven't found NLR to be an independent poor prognostic factor in GBM in our total cohort. However, in the subgroup of patients who completed Stupp protocol, a preoperative NLR > 7 correlated with a shorter overall survival. We haven't found any correlation of PLR with overall survival and progression-free survival. Robust studies on this subject are still scarce. Prospective and larger trials are needed to confirm or exclude NLR as a prognostic factor in GBM, but this could be a promising marker.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

References

- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144:646–674
- Donskov F (2013) Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Semin Cancer Biol* 23:200–207
- Templeton AJ, McNamara MG, Seruga B et al (2014) Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. doi:10.1093/jnci/dju124
- Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ (2013) The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol* 88:218–230
- Darefsky AS, King JT Jr., Dubrow R (2012) Adult glioblastoma multiforme survival in the temozolomide era: a population-based analysis of Surveillance, Epidemiology, and End Results registries. *Cancer* 118:2163–2172
- Gorlia T, van den Bent MJ, Hegi ME (2008) Nomograms for predicting survival of patients with newly diagnosed glioblastoma: prognostic factor analysis of EORTC and NCIC trial 26981 – 22981/CE.3. *Lancet Oncol* 9:29–38
- Lamborn KR, Chang SM, Prados MD (2004) Prognostic factors for survival of patients with glioblastoma: recursive partitioning analysis. *Neuro Oncol* 6:227–235
- Fekete B, Werlenius K, Örndal C, Rydenhag B (2015) Prognostic factors for glioblastoma patients—A clinical population-based study. *Acta Neurol Scand*. doi:10.1111/ane.12481
- Bambury RM, Teo MY, Power DG et al (2013) The association of pre-treatment neutrophil to lymphocyte ratio with overall survival in patients with glioblastoma multiforme. *J Neurooncol* 114:149–154
- McNamara MG, Lwin Z, Jiang H et al (2014) Factors impacting survival following second surgery in patients with glioblastoma in the temozolomide treatment era, incorporating neutrophil/lymphocyte ratio and time to first progression. *J Neurooncol* 117:147–152
- Han S, Liu Y, Li Q, Li Z, Hou H, Wu A (2015) Pre-treatment neutrophil-to-lymphocyte ratio is associated with neutrophil and T-cell infiltration and predicts clinical outcome in patients with glioblastoma. *BMC Cancer* 15:617–626
- Wang PF, Song HW, Cai HQ et al (2017) Preoperative inflammation markers and IDH mutation status predict glioblastoma patient survival. *Oncotarget*. doi:10.18632/oncotarget.15235
- Alexiou GA, Vartholomatos E, Voulgaris S (2013) Prognostic value of neutrophil-to-lymphocyte ratio in patients with glioblastoma. *J Neurooncol* 115:521–522
- Weller M, van den Bent M, Hopkins K et al (2014) EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *Lancet Oncol* 15:e395–e403
- Park CK, Kim JH, Nam DH et al (2013) A practical scoring system to determine whether to proceed with surgical resection in recurrent glioblastoma. *Neuro Oncol* 15:1096–1101
- Subeikshanan V, Dutt A, Basu D, Tejus MN, Maurya VP, Madhugiri VS (2016) A prospective comparative clinical study of peripheral blood counts and indices in patients with primary brain tumors. *J Postgrad Med* 62:86–90
- Wang W, Zhang L, Wang Z et al (2016) A three-gene signature for prognosis in patients with MGMT promoter-methylated glioblastoma. *Oncotarget*. doi:10.18632/oncotarget.11726
- Parsons DW, Jones S, Zhang X et al (2008) An integrated genomic analysis of human glioblastoma multiforme. *Science* 321:1807–1812
- Sanson M, Marie Y, Paris S et al (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J Clin Oncol* 27:4150–4154
- Kim W, Liao LM (2012) IDH mutations in human glioma. *Neurosurg Clin N Am* 23:471–480
- Chen JR, Yao Y, Xu HZ, Qin ZY (2016) Isocitrate dehydrogenase (IDH)1/2 mutations as prognostic markers in patients with glioblastomas. *Medicine (Baltimore)* 95:e2583
- Wang XW, Ciccarino P, Rossetto M et al (2014) IDH mutations: genotype-phenotype correlation and prognostic impact. *Biomed Res Int*. doi:10.1155/2014/540236
- Gravendeel LA, Kloosterhof NK, Bralten LB et al (2010) Segregation of non-p.R132H mutations in IDH1 in distinct molecular subtypes of glioma. *Hum Mutat* 31:E1186–99
- Louis D, Ohgaki H, Wiestler O et al (2016) WHO classification of tumours of the central nervous system. WHO, Geneva

25. Roxburgh CSD, McMillan DC (2010) Role of systemic inflammatory response in predicting survival in patients with primary operable cancer. *Future Oncol* 6:149–163
26. Fossati G, Ricevuti G, Edwards SW, Walker C, Dalton A (1999) Neutrophil infiltration into human gliomas. *Acta Neuropathol* 98:349–354
27. Zadora P, Dabrowski W, Czarko K et al (2015) Preoperative neutrophil-lymphocyte count ratio helps predict the grade of glial tumor—A pilot study. *Neurol Neurochir Pol* 49:41–44
28. Bhat T, Teli S, Rijal J et al (2013) Neutrophil to lymphocyte ratio and cardiovascular diseases—A review. *Expert Rev Cardiovasc Ther* 11:55–59
29. Belen E, Sungur A, Sungur MA, Erdogan G (2015) Increased neutrophil to lymphocyte ratio in patients with resistant hypertension. *J Clin Hypertens* 17:532–537
30. Lou M, Luo P, Tang R, Peng Y, Yu S, Huang W, He L (2015) Relationship between neutrophil-lymphocyte ratio and insulin resistance in newly diagnosed type 2 diabetes mellitus patients. *BMC Endocr Disord*. doi:[10.1186/s12902-015-0002-9](https://doi.org/10.1186/s12902-015-0002-9)
31. Vaziri ND, Pahl MV, Crum A, Norris K (2012) Effect of uremia on structure and function of immune system. *J Ren Nutr* 22:149–156
32. Fu H, Qin B, Hu Z, Ma N, Yang M, Wei T (2015) Neutrophil- and platelet-to-lymphocyte ratios are correlated with disease activity in rheumatoid arthritis. *Clin Lab* 61:269–273
33. Li L, Xia Y, Chen C, Cheng P, Peng C (2015) Neutrophil-lymphocyte ratio in systemic lupus erythematosus disease: a retrospective study. *Int J Clin Exp Med* 8:11026–11031
34. McKay LI, Cidlowski JA (2003) Physiologic and pharmacologic effects of corticosteroids. In: Kufe DW, Pollock RE, Weichselbaum RR et al (eds) *Holland-Frei cancer medicine*, 6th edn. BC Decker, Hamilton
35. Proctor MJ, Morrison DS, Talwar D et al (2011) A comparison of inflammation-based prognostic scores in patients with cancer. A Glasgow Inflammation Outcome study. *Eur J Cancer* 47:2633–2641
36. Partridge M, Fallon M, Bray C, McMillan D, Brown D, Laird B (2012) Prognostication in advanced cancer—A study examining an inflammation-based score. *J Pain Symptom Manage* 44:161–167
37. McMillan DC (2013) The systemic inflammation-based Glasgow prognostic score: a decade of experience in patients with cancer. *Cancer Treat Rev* 39:534–540
38. Kast RE, Scheuerle A, Wirtz CR, Karpel-Massler G, Halatsch ME (2011) The rationale of targeting neutrophils with dapsone during glioblastoma treatment. *Anticancer Agents Med Chem* 11:756–761

6 EFFECTS OF THE FUNCTIONAL *HOTAIR* RS920778 AND RS12826786 GENETIC VARIANTS IN GLIOMA SUSCEPTIBILITY AND PATIENT PROGNOSIS

Ana Xavier-Magalhães,^{1,2} Ana I. Oliveira,^{1,2} Joana Vieira de Castro,^{1,2} Marta Pojo,^{1,2} Céline S. Gonçalves,^{1,2} Tatiana Lourenço,^{1,2} Marta Viana-Pereira,^{1,2} Sandra Costa,^{1,2} Paulo Linhares,³ Rui Vaz,³ Rui Nabiço,⁴ Júlia Amorim,⁴ Afonso A. Pinto,⁵ Rui M. Reis,^{1,2,6} Bruno M. Costa^{1,2}

¹ Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; ² ICVS/3B's - PT Government Associate Laboratory, University of Minho, Braga/Guimarães, Campus de Gualtar, 4710-057 Braga, Portugal; ³ Department of Neurosurgery, Hospital S. João, Porto, Portugal; ⁴ Department of Oncology, Hospital de Braga, Braga, Portugal; ⁵ Department of Neurosurgery, Hospital de Braga, Braga, Portugal; ⁶ Molecular Oncology Research Center, Barretos Cancer Hospital, 1331 Barretos - SP - CEP 14.784-400, São Paulo, Brazil.

LABORATORY INVESTIGATION

Effects of the functional *HOTAIR* rs920778 and rs12826786 genetic variants in glioma susceptibility and patient prognosis

Ana Xavier-Magalhães^{1,2} · Ana I. Oliveira^{1,2} · Joana Vieira de Castro^{1,2} · Marta Pojo^{1,2} · Céline S. Gonçalves^{1,2} · Tatiana Lourenço^{1,2} · Marta Viana-Pereira^{1,2} · Sandra Costa^{1,2} · Paulo Linhares³ · Rui Vaz³ · Rui Nabico⁴ · Júlia Amorim⁴ · Afonso A. Pinto⁵ · Rui M. Reis^{1,2,6} · Bruno M. Costa^{1,2}

Received: 30 June 2016 / Accepted: 21 December 2016
© Springer Science+Business Media New York 2017

Abstract Abnormal expression of the long non-coding RNA HOX transcript antisense intergenic RNA (*HOTAIR*) is oncogenic in several human cancers, including gliomas. The *HOTAIR* single nucleotide polymorphisms (SNPs) rs920778 (C>T) and rs12826786 (C>T) present in the intronic enhancer and promoter regions of *HOTAIR*, respectively, are associated with expression, cancer susceptibility, and patient prognosis in some tumor types. However, the relevance of these *HOTAIR* SNPs has not been studied in glioma. Here, we report a case-control study comprising 177 Portuguese glioma patients and 199 cancer-free controls. All subjects were genotyped by PCR and

restriction fragment length polymorphism (RFLP). No statistically significant differences were found in the genotype or allele distributions of either rs920778 or rs12826786 between glioma patients and controls, suggesting these SNPs are not associated with glioma risk. No significant associations were found between rs920778 variants and *HOTAIR* expression levels, while rs12826786 CT genotype was associated with increased intratumoral *HOTAIR* RNA levels when compared to TT genotype (p -value=0.04). Univariate (Log-rank) and multivariate (Cox proportional) analyses showed both rs920778 CT and rs12826786 CT genotypes were significantly associated with longer overall survival of WHO grade III anaplastic oligodendroglioma patients. Our results suggest that *HOTAIR* SNPs rs920778 and rs12826786 do not play a significant role in glioma susceptibility, but may be important prognostic factors in anaplastic oligodendroglioma patients. Future studies are warranted to validate and expand these findings, and to further dissect the importance of these SNPs in glioma.

Ana Xavier-Magalhães and Ana I. Oliveira have contributed equally for this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11060-016-2345-0) contains supplementary material, which is available to authorized users.

✉ Bruno M. Costa
bfmcosta@med.uminho.pt

¹ Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

² ICVS/3B's - PT Government Associate Laboratory, University of Minho, Braga/Guimarães, Campus de Gualtar, 4710-057 Braga, Portugal

³ Department of Neurosurgery, Hospital S. João, Porto, Portugal

⁴ Department of Oncology, Hospital de Braga, Braga, Portugal

⁵ Department of Neurosurgery, Hospital de Braga, Braga, Portugal

⁶ Molecular Oncology Research Center, Barretos Cancer Hospital, 1331 Barretos-SP - CEP 14.784-400, São Paulo, Brazil

Keywords Glioma · *HOTAIR* · SNP · Prognosis · Rs920778 · Rs12826786

Introduction

The recent advent of new sequencing technologies and genome-wide analysis has indicated that the vast majority of the genome is transcribed into non-coding transcripts, and that only 2% of the genome accounts for protein-coding transcripts, highlighting the importance of better understanding the functions of non-protein coding genes [1, 2]. Among non-coding RNAs, those longer than 200 nucleotides—termed long non-coding RNAs (lncRNAs)—have emerged as highly attractive to cancer research due to their

ability to local or global regulation of gene expression via epigenetic, transcriptional or post-transcriptional mechanisms [3]. Indeed, several lncRNAs were found to have crucial roles in controlling cellular pathways and gene expression programs, thus impacting the oncogenic processes by presenting either oncogenic or tumor suppressive functions [4]. The HOX Transcript Antisense Intergenic RNA (*HOTAIR*) is a trans-acting lncRNA able to reprogram chromatin organization throughout the genome, as it is scaffold for the binding of Polycomb Repressive Complex (PRC2) in its 5' domain, while interacting on the 3' domain with LSD1/REST/CoREST complex [5, 6]. The oncogenic roles of *HOTAIR* have been extensively described in several tumor types, and its abnormal expression was associated with patient poor prognosis, tumor growth, aggressiveness, progression, recurrence, metastatic dissemination, invasion, and chemoresistance (reviewed in [7, 8]).

Gliomas are the most frequent primary malignant tumors of the central nervous system in adults, and are divided in 2 main histological subtypes, including astrocytomas and oligodendrogliomas [9]. Astrocytomas represent the vast majority of all diagnosed gliomas (about 70%), and are graded from I to IV according to the World Health Organization (WHO) [10]. Glioblastoma (GBM; astrocytoma WHO grade IV) is the most frequent and lethal, accounting for more than 50% of all glial tumor types [11], and is characterized by rapid growth and diffuse invasiveness of the adjacent brain parenchyma [10]. While presenting different molecular profiles resulting from distinct etiological mechanisms, no environmental factors have been firmly associated with the development of glioma, with exception of exposure to high-dose therapeutic radiation. Other putative causes may include familial aggregation, genetic syndromes, telomere maintenance and genetic polymorphisms [12–14]. The importance of inherited genetic variants in glioma risk has been

highly addressed in both candidate-gene and genome-wide association studies (GWAS) approaches, which revealed single nucleotide polymorphisms (SNPs) in cancer-related genes associated with glioma risk (e.g., *TERT*, *EGF*, *EGFR*, *TGF- β 1*, *CCDC26*, *CDKN2A/CDKN2B*, *PHLDB1* and *RTEL1* [15–18]).

Although *HOTAIR* expression has been reported as crucial in the carcinogenic process of many cancer types, and its abnormal expression was associated with patient poor prognosis, tumor grade, growth and invasion, and as a putative therapeutic target in glioma [19–21], few studies have focused on the significance of SNPs in *HOTAIR* affecting cancer risk [22–28]. Critically, no studies have evaluated the impact of *HOTAIR* SNPs in glioma risk and patient prognosis. In this context, we evaluated how previously described *HOTAIR* SNPs rs920778 and rs12826786 may associate with glioma susceptibility and patient prognosis.

Materials and methods

Study population

This case-control study comprised 177 Portuguese glioma patients from Hospital de Braga, Hospital de São João, and Hospital de Santo António, diagnosed between 2004 and 2015, and 199 cancer-free individuals randomly selected from blood donors as controls. All participants were of Caucasian ethnic background. Tumors were classified according to WHO grading system [29], and clinicopathological data is summarized in Table 1. The study was conducted according to institutional and European Union ethical standards, and all subjects ensured written informed consent to participate in research studies.

Table 1 Clinicopathologic features of glioma patients and cancer-free controls

Groups (WHO grade)	Number of cases	Age, y		Gender	
		mean $\pm$ SD	p-value	M/F ratio	p-value
Controls	199	46.4 $\pm$ 14.0	N/A	1.88	N/A
Gliomas (II–IV)	177	57.7 $\pm$ 12.4	$p \leq 0.001$	1.68	$p = 0.598$
Astrocytoma (II–IV)	137	58.3 $\pm$ 12.1	$p \leq 0.001$	1.85	$p = 0.945$
Diffuse astrocytoma (II)	7	53.1 $\pm$ 13.3	$p = 0.153$	1.33	$p = 0.656$
Anaplastic astrocytoma (III)	5	56.0 $\pm$ 9.9	$p = 0.080$	0.25	$p = 0.037$
Glioblastoma (IV)	118	59.0 $\pm$ 12.1	$p \leq 0.001$	2.11	$p = 0.654$
Gliosarcoma (IV)	7	53.7 $\pm$ 12.5	$p = 0.131$	1.33	$p = 0.656$
Oligodendroglioma (II–III)	38	55.9 $\pm$ 13.5	$p \leq 0.001$	1.24	$p = 0.238$
Oligodendroglioma (II)	8	46.5 $\pm$ 14.3	$p = 0.680$	0.33	$p = 0.020$
Anaplastic oligodendroglioma (III)	30	58.5 $\pm$ 12.3	$p \leq 0.001$	1.73	$p = 0.831$
Anaplastic oligoastrocytoma (III)	2	49.5 $\pm$ 19.1	$p = 0.696$	1.00	$p = 0.652$

F female, M male, N/A not Applicable, SD standard deviation

SNP genotyping

Genomic DNA was collected from peripheral blood leukocytes by either proteinase K/chloroform/isopropanol treatment or Citogene® DNA isolation kit (Citomed). *HOTAIR* SNPs rs920778 (C>T) and rs12826786 (C>T) were genotyped through PCR-based restriction fragment length polymorphism (RFLP). Fifty ng of DNA were used for PCR amplification with KAPA Taq DNA Polymerase (Kapa Biosystems). Primers sequences (following previous publications [22, 23]) and PCR conditions are listed in Supplementary Table 1. Ten µL of PCR product were enzymatically digested at 37°C for 5 or 30 min with Fast-Digest MspI (Thermo Scientific, rs920778) or FastDigest BglII (Thermo Scientific, rs12826786), respectively. Digestion products were resolved in 4% agarose gel stained with GreenSafe Premium (Nzytech). For quality control, 20 cases were confirmed by Sanger Sequencing, and the reproducibility was 100%.

Intratumoral *HOTAIR* expression levels

Total RNA was collected from the available glioma tissues from patients from Hospital de Braga using TRIzol method (Invitrogen), and cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Gene expression levels were assessed by quantitative real-time PCR (qPCR; Kapa Biosystems) with the following set of primers: Sense- CAGTGGGGAAGTCTGACTCG and Antisense- GTGCCCTGGTGCTCTTACC, and normalized to the expression of *HPRT1* (Sense- TGAGGATTTGGAAAGGTGT; Antisense- GAGCACACAGAGGGC TACAA).

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (IBM SPSS Statistics, IBM®). The different allele and genotype frequencies in glioma patients and cancer-free controls were compared by Chi square test, and nonparametric Wilcoxon-Mann Whitney test was used to compare age and sex distributions between these groups. Hardy-Weinberg equilibrium was evaluated by Chi square test, comparing the expected and observed genotype frequencies of *HOTAIR* SNPs in control group. Linkage disequilibrium (LD) was calculated with the SHEsis program [30]. The intratumoral expression levels of *HOTAIR* were compared between genotypes using Mann-Whitney test. Odds ratio (OR) and 95% confidence intervals (CI) for assessing differences in risk and survival were calculated by univariate and a Cox multivariate logistic regression (adjusted for patient age and sex). Effect sizes were measured using Cohen's *d*

values, and large effect sizes were considered for $d \geq 0.8$ [31]. No multiple comparisons corrections were performed. Patients' overall survival was assessed by Kaplan-Meier survival curves, and the log-rank test was used to evaluate the differences. Statistical significance was considered for p -values < 0.05 .

Results

Subject characteristics

A total of 376 Portuguese subjects (177 glioma patients and 199 cancer-free control individuals) were genotyped to determine possible associations between *HOTAIR* rs920778 and rs12826786 polymorphisms and glioma risk or prognosis. The clinicopathological features of the controls and cases are shown in Table 1. Statistical analysis of age (controls: 27–85 years; gliomas: 20–83 years) and sex distribution between control and all glioma cases showed significant differences for age (p -value ≤ 0.001), but not for sex (p -value = 0.598; Table 1).

Allelic frequencies and genotype distributions of *HOTAIR* SNPs

The genotype and allele frequencies of *HOTAIR* rs920778 and rs12826786 polymorphisms in controls and glioma cases are shown in Table 2. The frequencies of TT, CT, and CC genotypes of rs920778 were 45.2, 42.2, and 12.6% in cancer-free controls, and 46.3, 40.1, and 13.6% in glioma patients, respectively. The allelic frequencies of T allele were 66.3% for controls and 66.4% for glioma. Regarding rs12826786 polymorphism, the frequencies of CC, CT, and TT genotypes were 47.2, 42.2, and 10.6% in controls, and 47.5, 43.5, and 9.0% in glioma cases, respectively, being the C allele the most representative in the control group (68.3%) and in glioma patients (69.2%). The distribution of rs920778 and rs12826786 allele frequencies in the control group was in Hardy-Weinberg equilibrium (p -value = 0.750 and p -value = 0.935, respectively). Interestingly, these two polymorphisms were in strong linkage disequilibrium ($D' = 0.94$ and $r^2 = 0.79$) in our datasets.

Association between *HOTAIR* SNPs and glioma risk

To define whether there was significantly increased glioma susceptibility due to the presence of particular *HOTAIR* rs920778 and rs12826786 genotypes, univariate (data not shown) and multivariate (Table 2) analyses were performed in all glioma and GBM patients, the most frequent subtype in our series ($n = 118$). No significant associations between these polymorphic variants and the risk for developing

Table 2 Multivariate logistic regression analysis of the association between rs920778 and rs12826786 polymorphisms and glioma susceptibility by WHO grades

Polymorphism	Control	Glioma (WHO grade II-IV)	OR (95% CI) ^a	Glioblastoma (WHO grade IV)	OR (95% CI) ^a
rs920778					
Genotypes					
TT	90	82	–	54	–
CT	84	71	0.969 (0.602–1.561)	45	0.927 (0.537–1.601)
CC	25	24	1.060 (0.528–2.127)	14	0.867 (0.384–1.961)
CC + CT	109	95	0.990 (0.635–1.545)	59	0.913 (0.547–1.522)
Alleles					
T	264	235	–	153	–
C	134	119	1.013 (0.727–1.412)	73	0.925 (0.630–1.359)
Age			1.063 (1.045–1.081)		1.071 (1.050–1.092)
Sex					
Male	130	111	–	76	–
Female	69	66	1.005 (0.632–1.598)	37	0.716 (0.414–1.239)
rs12826786					
Genotypes					
CC	94	84	–	57	–
CT	84	77	1.040 (0.651–1.661)	48	0.966 (0.565–1.654)
TT	21	16	0.837 (0.384–1.825)	8	0.533 (0.202–1.411)
TT + CT	105	93	0.998 (0.640–1.556)	56	0.872 (0.523–1.454)
Alleles					
C	272	245	–	162	–
T	126	109	0.959 (0.683–1.346)	64	0.821 (0.552–1.221)
Age	–	–	1.063 (1.045–1.081)	–	1.072 (1.051–1.093)
Sex					
Male	130	111	–	76	–
Female	69	66	1.000 (0.628–1.591)	37	0.701 (0.403–1.218)

Boldfaced values indicate statistical significant differences at 5% level

^aOdds ratio (OR) with 95% confidence intervals (CI), adjusted for age (as a continuous variable) and sex

glioma or GBM were found (p -value > 0.05). As expected, in the multivariate analyses, increased age was associated with increased risk for developing glioma (OR 1.063; 95% CI 1.045–1.081) or GBM (OR 1.071; 95% CI 1.050–1.092; Table 2).

Association between *HOTAIR* SNPs and gene expression levels in glioma specimens

The expression of *HOTAIR* was evaluated in 34 glioma tumor tissues (freshly collected adequately for RNA analyses) to understand whether the rs920778 and rs12826786 SNPs may be associated with *HOTAIR* mRNA levels (Fig. 1). For rs920778, there were no statistically significant differences between CC, TT and CT genotypes and *HOTAIR* expression (Fig. 1a). In contrast, glioma subjects with the rs12826786 CT genotype had significantly higher tumor *HOTAIR* RNA levels than those with the TT genotype (Fig. 1b, p -value=0.04).

Prognostic value of *HOTAIR* SNPs in glioma patients

We next investigated the association between these *HOTAIR* polymorphisms and patients' overall survival in WHO grade IV GBM ($n=69$) and WHO grade III anaplastic oligodendroglioma tumors ($n=18$) with available follow-up data.

No statistically significant associations were found between rs920778 or rs12826786 polymorphisms and survival of GBM patients both by univariate (log rank test p -value=0.458 and p -value=0.306, respectively) and multivariate analyses adjusted for patients' age and sex (Cox regression model p -values > 0.05; data not shown).

In contrast, univariate analyses for both SNPs in anaplastic oligodendroglioma patients showed that carriers of CT genotypes present longer overall survival than CC patients (p -value=0.029 and p -value ≤ 0.001, for rs920778 and rs12826786, respectively; Fig. 2) or when compared to all other patients (CT vs CC+TT;

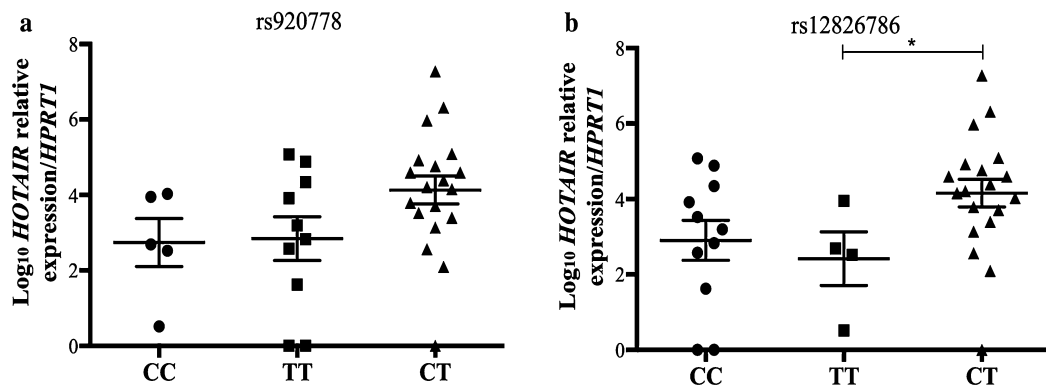


Fig. 1 *HOTAIR* expression levels in glioma tissues grouped by rs920778 and rs12826786 genotypes. Within rs920778 genotypes (a), *HOTAIR* expression levels are not statistically different (CC n=5, TT n=10, CT n=19), whereas in rs12826786 polymorphism (b), patients presenting CT (n=19) genotype have increased levels of

HOTAIR in comparison to TT patients (n=4), but not to CC patients (n=11). The expression levels were normalized to *HPRT1* expression using the $2^{-\Delta C_t}$ method, transformed in logarithmic ($\log_{10}$) scale, and are represented by mean $\pm$ SEM. * p -value=0.04

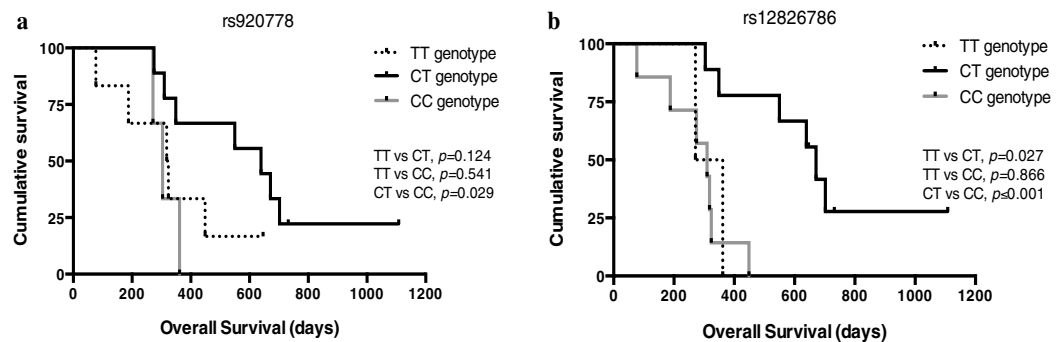


Fig. 2 Effect of *HOTAIR* rs920778 and rs12826786 polymorphisms in the survival of WHO grade III anaplastic oligodendroglioma patients. Kaplan-Meier survival curves indicate that a patients with CT genotype (n=9) in rs920778 locus of *HOTAIR* present statistically significant longer overall survival than patients carrying the CC genotype (n=3; Log-rank test, p -value=0.029), but not the TT genotype (n=6; Log-rank test, p -value=0.124). No statistically significant associations were found between the TT and the

CC genotypes (p -value=0.541). b Patients harboring the CT genotype (n=9) in rs12826786 SNP have statistically significant longer overall survival than patients with the CC genotype (n=7; Log-rank test, p -value $\leq$ 0.001) and the TT genotype (n=2; Log-rank test, p -value=0.032). No statistically significant associations were found between the TT and the CC genotypes (p -value=0.866). Tick marks indicate censored data

p -value=0.043 and p -value $\leq$ 0.001, for rs920778 and rs12826786, respectively; data not shown). Additionally for rs12826786, CT genotype patients also presented longer overall survival when compared to TT patients (p -value=0.027; Fig. 2b).

Accordingly, Cox multivariate analysis showed that, for both SNPs, patients with the CT genotype present statistically significant longer overall survival than CC patients, independently of age and sex (p -value=0.032 and p -value=0.010, for rs920778 and rs12826786, respectively; Table 3). Moreover, for rs12826786, the CT genotype also conferred longer overall survival than TT

genotype patients (p -value=0.032; Table 3) or when compared to all other genotypes (p -value=0.006; Table 3).

Discussion

The discovery of genetic factors associated with glioma susceptibility is required for a deeper understanding of this devastating disease, and to develop novel therapeutic approaches. *HOTAIR* is a known oncogenic lncRNA in several tumor types (reviewed in [7, 8]) including gliomas [15–17]. The roles of *HOTAIR* have been widely reported

Table 3 Multivariate Cox regression analysis of the association between rs920778 and rs12826786 polymorphisms and survival in WHO grade III anaplastic oligodendroglioma patients

Polymorphism	Number of cases	p-value	HR (95% CI) ^b	Cohen's <i>d</i>
rs920778				
Genotypes				
CT	9	0.094	–	–
CC	3	0.032	6.292 (1.175–33.69)	1.014 ^a
TT	6	0.267	2.320 (0.526–10.24)	0.464
CC + TT	9	0.070	3.264 (0.909–11.72)	0.652
Age		0.187	1.046 (0.978–1.119)	0.025
Sex				
Male	13	–	–	–
Female	5	0.236	2.305 (0.578–9.188)	0.460
rs12826786				
Genotypes				
CT	9	0.022	–	–
CC	7	0.010	12.34 (1.837–82.93)	1.386 ^a
TT	2	0.032	9.188 (1.209–69.81)	1.223 ^a
CC + TT	9	0.006	10.79 (1.998–58.24)	1.311 ^a
Age		0.392	1.026 (0.968–1.087)	0.014
Sex				
Male	13	–	–	–
Female	5	0.754	0.791 (0.182–3.437)	–0.129

Bold-faced values indicate statistical significant differences at 5% level

^aValues that exceed Cohen's *d* convention for a large effect size ($d \geq 0.8$)

^bHazard ratio (HR) with 95% confidence intervals (CI), adjusted for age (as a continuous variable) and sex

in cancer, but epidemiological studies evaluating tumor susceptibility conferred by genetic polymorphisms in its locus have not been extensively assessed [18–22]. Indeed, to the best of our knowledge, the current study is the first to evaluate associations between *HOTAIR* SNPs (rs920778 and rs12826786) and glioma susceptibility and prognosis. We have analyzed a Portuguese population consisting of 177 glioma patients and 199 cancer-free controls. Our results showed that the allelic frequencies of *HOTAIR* SNPs rs920778 and rs12826786 were not significantly different between glioma patients and cancer-free controls. Importantly, for both polymorphisms, the genotypes frequencies in cancer-free controls are in agreement with those reported by the HapMap Project for subjects of European ancestry (HapMap-CEU, data not shown; <http://hapmap.ncbi.nlm.nih.gov/>). Considering risk assessments, our results showed no significant associations between *HOTAIR* rs920778 or rs12826786 and susceptibility for any WHO grade glioma. Previous studies on the contribution of rs920778 to gastric cancer in a Turkish population [25] also showed

no differences between patients and cancer-free controls; however, studies in other tumor types testing the importance of rs920778 or rs12826786 in the Chinese population have shown different allele frequencies between patients and cancer-free controls [22–24]. It was also showed previously that rs920778 variants do not influence gastric cancer susceptibility [25], whereas others have showed statistically significant associations between these SNPs and risk to develop gastric cardia adenocarcinoma [22], esophageal squamous cell carcinoma (ESCC) [23], breast cancer [26–28] or gastric cancer [24]. Such discrepancies might be attributed to the different genetic backgrounds of the studied populations, different sample sizes, and/or to the variety of tumor types analyzed. Indeed, the expression patterns, the regulatory mechanisms, and the functions of lncRNAs are highly tissue-specific [32]. Because SNPs differentially influence the regulation of lncRNAs [33], in particular the SNPs in this study [22–24], it is possible that these SNPs may present distinct relevance in the context of different tumor types.

Since rs920778 and rs12826786 *HOTAIR* SNPs have been previously reported to be located in an intronic enhancer or in the promoter, respectively, we sought to understand if the different genotypes influence the expression levels of *HOTAIR* in glioma tissues. Our results showed that patients with the rs12826786 CT genotype presented increased intratumoral levels of *HOTAIR*, compared to TT genotype but not CC, whereas in rs920778 no differences were found. Previous reports showed increased levels of *HOTAIR* in TT homozygous patients with gastric cancer or gastric cardia adenocarcinoma for rs920778 [24] or rs12826786 [22], respectively, again highlighting tumor type-specific roles of SNPs.

Given that *HOTAIR* expression is a known negative prognostic factor in glioma [19], we evaluated the importance of rs920778 or rs12826786 genotypes in the overall survival of the glioma patients from our dataset. We did not find any associations between rs920778 and rs12826786 and overall survival for GBM patients (data not shown). Conversely, anaplastic oligodendroglioma patients presenting the CT genotype for rs920778 or rs12826786 had significantly longer overall survivals, both in univariate and age- and sex-adjusted multivariate analyses (Fig. 2; Table 3). Interestingly, in our GBM dataset, the TT genotype is the least frequent ($n = 5/69$), and these patients presented a tendency for shorter overall survival in comparison to either CC or CT patients. Concordantly, a report in gastric cardia adenocarcinoma [22] has shown for the rs12826786 SNP that TT genotype is the least frequent and that these patients also present a tendency to shorter overall survival in comparison to CT or CC, while presenting no overall survival differences for CC or CT patients. It is important to highlight that our survival analyses are based on a small

cohort of patients ($n=18$ for WHO grade III anaplastic oligodendroglioma and $n=69$ for WHO grade IV GBM), for which we had available follow-up information. Nonetheless, our findings that rs920778 and rs12826786 CT genotypes are associated with longer survival of WHO grade III anaplastic oligodendroglioma patients are integrally supported by complementary statistical approaches, including Log-rank tests (which take into consideration sample size), multivariate Cox regression models adjusted for potential confounding effects (like patient age and sex), as well as calculations of Hazard Ratios and Cohen's d values (which relate to the effect size, and are independent of sample size; Fig. 2; Table 3). These findings suggest putative roles of these *HOTAIR* SNPs in the survival of anaplastic oligodendroglioma patients, warranting further validation by independent studies with larger datasets.

In conclusion, our findings add to the body of knowledge surrounding genetic polymorphisms as putative players affecting glioma risk or prognosis. We found that *HOTAIR* rs920778 and rs12826786 do not seem to play any major role in genetic susceptibility of glioma in the Portuguese population. In contrast, *HOTAIR* rs12826786 presents genotype specific effect on tumoral *HOTAIR* expression levels, and both SNPs showed prognostic significance in the Portuguese anaplastic oligodendroglioma population. Therefore, *HOTAIR* rs920778 and rs12826786 may be useful biomarkers to predict the outcome of WHO grade III anaplastic oligodendroglioma patients. Of note, these two SNPs were in strong linkage disequilibrium in the glioma population, which could partly explain the similar results obtained for both SNPs relating to risk and prognostic analysis. Further studies are required to validate and expand our results to populations with different genetic backgrounds to better understand the broader importance of these SNPs in gliomas. Taking advantage of genotyping projects (e.g., HapMap and the 1000genomes.org), it may be interesting to identify and study additional *HOTAIR* polymorphisms, which may have functional consequences and play a role in glioma pathophysiology.

Acknowledgements The authors would like to acknowledge the funding agencies that supported this work: Fundação para a Ciência e Tecnologia (PTDC/SAU-GMG/113795/2009 and IF/00601/2012 to B.M.C.; SFRH/BD/88220/2012 to A.X.M.; SFRH/BD/52287/2013 to A.I.O.; SFRH/BD/88121/2012 to J.V.C.; SFRH/BD/81042/2011 to M.P.; SFRH/BD/92786/2013 to C.S.G.; SFRH/BD/51996/2012 to T.L.; SFRH/BPD/104290/2014 to M.V.P.; PTDC/SAU-ONC/115513/2009 to R.M.R., Fundação Calouste Gulbenkian (B.M.C.), Liga Portuguesa Contra o Cancro (B.M.C.) and Inter-University Doctoral Programme in Ageing and Chronic Disease (PhDOC; to A.X.M. and A.I.O.). Project co-financed by Programa Operacional Regional do Norte (ON.2—O Novo Norte), Quadro de Referência Estratégico Nacional (QREN), Fundo Europeu de Desenvolvimento Regional (FEDER). The authors would like to extend their appreciation to all the patients and control subjects that kindly provided the biological samples for this study.

Compliance with ethical standards

Conflict of interest The authors disclose no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human and animal participants This article does not contain any studies with animals performed by any of the authors.

References

1. Consortium EP (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* 489(7414):57–74. doi:10.1038/nature11247
2. Consortium EP, Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR, Margulies EH, Weng Z, Snyder M, Dermitzakis ET et al (2007) Identification and analysis of functional elements in 1 % of the human genome by the ENCODE pilot project. *Nature* 447(7146):799–816. doi:10.1038/nature05874
3. Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. *Nature reviews Genetics* 10(3):155–159. doi:10.1038/nrg2521
4. Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, Khalil AM, Zuk O, Amit I, Rabani M et al (2010) A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 142(3):409–419. doi:10.1016/j.cell.2010.06.040
5. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E et al (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129(7):1311–1323. doi:10.1016/j.cell.2007.05.022
6. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY (2010) Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329(5992):689–693. doi:10.1126/science.1192002
7. Hajjari M, Salavaty A (2015) *HOTAIR*: an oncogenic long non-coding RNA in different cancers. *Cancer Biol Med* 12(1):1–9. doi:10.7497/j.issn.2095-3941.2015.0006
8. Wu Y, Zhang L, Wang Y, Li H, Ren X, Wei F, Yu W, Wang X, Zhang L, Yu J et al (2014) Long noncoding RNA *HOTAIR* involvement in cancer. *Tumour Biol* 35(10):9531–9538. doi:10.1007/s13277-014-2523-7
9. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P, Ellison DW (2016) The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol (Berl)* 131(6):803–820. doi:10.1007/s00401-016-1545-1
10. Kleihues P, Sobin LH (2000) World Health Organization classification of tumors. *Cancer* 88(12):2887
11. Lino MM, Merlo A (2010) PI3Kinase signaling in glioblastoma. *J Neurooncol*. doi:10.1007/s11060-010-0442-z
12. Ohgaki H, Kleihues P (2005) Epidemiology and etiology of gliomas. *Acta Neuropathol (Berl)* 109(1):93–108. doi:10.1007/s00401-005-0991-y
13. Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, Kruchko C, McCarthy BJ, Rajaraman P,

- Schwartzbaum JA et al (2008) Brain tumor epidemiology: consensus from the brain tumor epidemiology consortium. *Cancer* 113(7 Suppl):1953–1968. doi:[10.1002/cncr.23741](https://doi.org/10.1002/cncr.23741)
14. Walsh KM, Wiencke JK, Lachance DH, Wiemels JL, Molinaro AM, Eckel-Passow JE, Jenkins RB, Wrensch MR (2015) Telomere maintenance and the etiology of adult glioma. *Neuro-oncol*. doi:[10.1093/neuonc/nov082](https://doi.org/10.1093/neuonc/nov082)
 15. Costa BM, Viana-Pereira M, Fernandes R, Costa S, Linhares P, Vaz R, Pinheiro C, Lima J, Soares P, Silva A et al (2011) Impact of EGFR genetic variants on glioma risk and patient outcome. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive*. *Int Soc Cell* 20(12):2610–2617. doi:[10.1158/1055-9965.EPI-11-0340](https://doi.org/10.1158/1055-9965.EPI-11-0340)
 16. Vieira de Castro J, Goncalves CS, Costa S, Linhares P, Vaz R, Nabico R, Amorim J, Viana-Pereira M, Reis RM, Costa BM (2015) Impact of TGF-beta1 -509C/T and 869 T/C polymorphisms on glioma risk and patient prognosis. *Tumour Biology* 36(8):6525–6532. doi:[10.1007/s13277-015-3343-0](https://doi.org/10.1007/s13277-015-3343-0)
 17. Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A, Pardal F, Suriano G, Machado JC, Lopes JM et al (2007) Association between functional EGF+ 61 polymorphism and glioma risk. *Clin Cancer Res* 13(9):2621–2626. doi:[10.1158/1078-0432.CCR-06-2606](https://doi.org/10.1158/1078-0432.CCR-06-2606)
 18. Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, Pekmezci M, Schwartzbaum JA, Turner MC, Walsh KM et al (2014) The epidemiology of glioma in adults: a “state of the science” review. *Neuro-oncol* 16(7):896–913. doi:[10.1093/neuonc/nou087](https://doi.org/10.1093/neuonc/nou087)
 19. Zhang JX, Han L, Bao ZS, Wang YY, Chen LY, Yan W, Yu SZ, Pu PY, Liu N, You YP et al (2013) HOTAIR, a cell cycle-associated long noncoding RNA and a strong predictor of survival, is preferentially expressed in classical and mesenchymal glioma. *Neuro-oncol* 15(12):1595–1603. doi:[10.1093/neuonc/not131](https://doi.org/10.1093/neuonc/not131)
 20. Zhang K, Sun X, Zhou X, Han L, Chen L, Shi Z, Zhang A, Ye M, Wang Q, Liu C et al (2015) Long non-coding RNA HOTAIR promotes glioblastoma cell cycle progression in an EZH2 dependent manner. *Oncotarget* 6(1):537–546
 21. Zhou X, Ren Y, Zhang J, Zhang C, Zhang K, Han L, Kong L, Wei J, Chen L, Yang J et al (2015) HOTAIR is a therapeutic target in glioblastoma. *Oncotarget* 6(10):8353–8365
 22. Guo W, Dong Z, Bai Y, Guo Y, Shen S, Kuang G, Xu J (2015) Associations between polymorphisms of HOTAIR and risk of gastric cardia adenocarcinoma in a population of north China. *Tumour Biol* 36(4):2845–2854. doi:[10.1007/s13277-014-2912-y](https://doi.org/10.1007/s13277-014-2912-y)
 23. Zhang X, Zhou L, Fu G, Sun F, Shi J, Wei J, Lu C, Zhou C, Yuan Q, Yang M (2014) The identification of an ESCC susceptibility SNP rs920778 that regulates the expression of lncRNA HOTAIR via a novel intronic enhancer. *Carcinogenesis* 35(9):2062–2067. doi:[10.1093/carcin/bgu103](https://doi.org/10.1093/carcin/bgu103)
 24. Pan W, Liu L, Wei J, Ge Y, Zhang J, Chen H, Zhou L, Yuan Q, Zhou C, Yang M (2015) A functional lncRNA HOTAIR genetic variant contributes to gastric cancer susceptibility. *Mol Carcinog*. doi:[10.1002/mc.22261](https://doi.org/10.1002/mc.22261)
 25. Bayram S, Ulger Y, Sumbul AT, Kaya BY, Rencuzogullari A, Genc A, Sevgiler Y, Bozkurt O, Rencuzogullari E (2015) A functional HOTAIR rs920778 polymorphism does not contribute to gastric cancer in a Turkish population: a case-control study. *Fam Cancer*. doi:[10.1007/s10689-015-9813-0](https://doi.org/10.1007/s10689-015-9813-0)
 26. Bayram S, Sumbul AT, Batmaci CY, Genc A (2015) Effect of HOTAIR rs920778 polymorphism on breast cancer susceptibility and clinicopathologic features in a Turkish population. *Tumour Biol* 36(5):3863–3870. doi:[10.1007/s13277-014-3028-0](https://doi.org/10.1007/s13277-014-3028-0)
 27. Bayram S, Sumbul AT, Dadas E (2015) A functional HOTAIR rs12826786 C > T polymorphism is associated with breast cancer susceptibility and poor clinicopathological characteristics in a Turkish population: a hospital-based case-control study. *Tumour Biol*. doi:[10.1007/s13277-015-4430-y](https://doi.org/10.1007/s13277-015-4430-y)
 28. Yan R, Cao J, Song C, Chen Y, Wu Z, Wang K, Dai L (2015) Polymorphisms in lncRNA HOTAIR and susceptibility to breast cancer in a Chinese population. *Cancer Epidemiol* 39(6):978–985. doi:[10.1016/j.canep.2015.10.025](https://doi.org/10.1016/j.canep.2015.10.025)
 29. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P (2007) The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol (Berl)* 114(2):97–109. doi:[10.1007/s00401-007-0243-4](https://doi.org/10.1007/s00401-007-0243-4)
 30. Shi YY, He L (2005) SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 15(2):97–98. doi:[10.1038/sj.cr.7290272](https://doi.org/10.1038/sj.cr.7290272)
 31. Cohen J (1988) *Statistical power analysis for the behavioral sciences*. 2nd edn. Lawrence Erlbaum Associates, Hillsdale
 32. Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS (2008) Specific expression of long noncoding RNAs in the mouse brain. *Proc Natl Acad Sci USA* 105(2):716–721. doi:[10.1073/pnas.0706729105](https://doi.org/10.1073/pnas.0706729105)
 33. Wu H, Zheng J, Deng J, Hu M, You Y, Li N, Li W, Lu J, Zhou Y (2013) A genetic polymorphism in lincRNA-uc003opf.1 is associated with susceptibility to esophageal squamous cell carcinoma in Chinese populations. *Carcinogenesis* 34(12):2908–2917. doi:[10.1093/carcin/bgt252](https://doi.org/10.1093/carcin/bgt252)

7 REPLICATION OF GWAS IDENTIFIES *RTEL1*, *CDKN2A/B*, AND *PHLDB1* SNPS AS RISK FACTORS IN PORTUGUESE GLIOMAS PATIENTS

Marta Viana-Pereira,^{1,2} Daniel Antunes Moreno,³ Paulo Linhares,^{4,5} Júlia Amorim,⁶ Rui Nabicho,⁶ Sandra Costa,^{1,2} Rui Vaz,^{4,5} Rui Manuel Reis,^{1,2,3}

¹School of Medicine, Life and Health Sciences Research Institute (ICVS), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; ²ICVS/3B's - PT Government Associate Laboratory, Minho, Portugal; ³Barretos Cancer Hospital, Molecular Oncology Research Center, Barretos, SP, Brazil; ⁴Department of Neurosurgery, Hospital S. João, Porto, Portugal; ⁵Faculty of Medicine, University of Porto, Porto, Portugal; ⁶Department of Oncology, Hospital de Braga, Braga, Portugal.



Replication of GWAS identifies *RTEL1*, *CDKN2A/B*, and *PHLDB1* SNPs as risk factors in Portuguese gliomas patients

Marta Viana-Pereira^{1,2} · Daniel Antunes Moreno³ · Paulo Linhares^{4,5} · Júlia Amorim⁶ · Rui Naboço⁶ · Sandra Costa^{1,2} · Rui Vaz^{4,5} · Rui Manuel Reis^{1,2,3}

Received: 2 August 2019 / Accepted: 31 October 2019
© Springer Nature B.V. 2019

Abstract

Diffuse gliomas are the most common malignant primary brain tumors and remain incurable. A better knowledge of the tumor etiology is required. Specific single nucleotide polymorphisms (SNPs) rs4977756 (*CDKN2A/B*), rs6010620 (*RTEL1*), rs498872 (*PHLDB1*), rs2736100 (*TERT*), and rs4295627 (*CCDC26*) have been associated with glioma susceptibility and are potential risk biomarkers. This study aimed to analyze five SNPs associated with glioma susceptibility, in the Portuguese population. SNPs were genotyped using the Sequenom MassARRAY platform in 127 gliomas and 180 controls. Unconditional logistic regression models were used to calculate odds ratio (OR) and 95% confidence intervals. The false-positive report probability was also assessed. The associations between polymorphisms and survival were evaluated using the log-rank test. It was found that the AG and GG genotypes of the rs4977756 (*CDKN2A/B*) were associated with an increased risk of gliomas (OR 1.85 and OR 2.38) and glioblastomas (OR 2.77 and OR 3.94). The GA genotype of the rs6010620 (*RTEL1*) was associated with a decreased risk of glioblastomas (OR 0.45). We also observed that the GA genotype of the rs498872 (*PHLDB1*) was associated with an increased risk of gliomas (OR 2.92) and glioblastomas (OR 2.39). No significant risk associations were found for the rs2736100 (*TERT*) and rs4295627 (*CCDC26*). In addition, the genotype AA of the rs498872 (*PHLDB1*) was associated with poor overall survival of gliomas patients (AA vs. GA, $p=0.037$). The rs6010620 (*RTEL1*), rs4977756 (*CDKN2A/B*), and rs498872 (*PHLDB1*) are associated with glioma risk in the Portuguese population and these data may contribute to understanding gliomas etiology.

Keywords Glioma risk · SNPs · GWAS · *RTEL1* · *CDKN2A/B* · *PHLDB1*

Abbreviations

A	Adenine	CCDC26	Coiled-coil domain containing 26
C	Cytosine	CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
		CI	Confidence interval

✉ Rui Manuel Reis
rreis@med.uminho.pt

Marta Viana-Pereira
martavianap@gmail.com

Daniel Antunes Moreno
daniellmoreno@gmail.com

Paulo Linhares
paulojlinhares@yahoo.com

Júlia Amorim
juliaamorim1@gmail.com

Rui Naboço
rnabico@hotmail.com
https://www.hospitaldebraga.pt/servicos/especialidades

Sandra Costa
sandracosta@med.uminho.pt

Rui Vaz
ruimcvaz@gmail.com

¹ School of Medicine, Life and Health Sciences Research Institute (ICVS), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

² ICVS/3B's - PT Government Associate Laboratory, Minho, Portugal

³ Barretos Cancer Hospital, Molecular Oncology Research Center, Barretos, SP, Brazil

⁴ Department of Neurosurgery, Hospital S. João, Porto, Portugal

⁵ Faculty of Medicine, University of Porto, Porto, Portugal

⁶ Department of Oncology, Hospital de Braga, Braga, Portugal

Published online: 12 November 2019

Springer

FPRP	False-positive report probability
G	Guanine
GWAS	Genome-wide association studies
OR	Odds ratio
OS	Overall survival
PHLDB1	Pleckstrin Homology-Like Domain Family B member 1
<i>RTEL1</i>	Regulator of telomere elongation helicase 1
SNPs	Single nucleotide polymorphisms
T	Thymine
TERT	Telomerase reverse transcriptase
WHO	World Health Organization

Background

Gliomas are the most common primary tumors of the central nervous system (CNS), with an annual incidence of nearly 6 per 100,000 in the USA [1–4]. In Portugal, gliomas are the 13th most common neoplasms, with an estimated number of 1225 new cases, and estimated mortality of 1042 new cases in 2018 [https://gco.iarc.fr]. Currently, gliomas are classified according to histologic and molecular features and growth patterns associated with clinical behavior [5]. Diffuse gliomas are characterized by infiltrative growth in the CNS parenchyma and based on mitotic activity markers, necrosis, microvascular proliferation, malignancy degrees are attributed; namely, World Health Organization (WHO) grades II, III and IV (glioblastoma) [2]. The mutational status of isocitrate dehydrogenase genes (*IDH1* and/or *IDH2*), deletion of short and long arms of chromosomes 1 and 19, and mutation of the *TERT* promoter region are important prognostic factors [4–8].

The median survival of diffuse glioma patients varies from 1.25 years (primary glioblastoma, *IDH*-wild type) to 8.0 years (diffuse gliomas grade II and III, *IDH*-mutant with 1p19q co-deletion) [5]. Low-grade diffuse gliomas usually progress to glioblastoma, one of the most aggressive and lethal tumors known. Besides extensive studies in the last decades, diffuse gliomas remain incurable, requiring better knowledge regarding prognostic/predictive molecular biomarkers that contribute to understanding the etiology of disease and risk stratification for improvements in therapy [7, 9, 10].

The environmental causes involved in gliomas development are largely unrecognized, with the exception of ionizing radiation that is a well-known etiological factor [11]. An increased risk of glioma is also associated with a family history of brain tumors and inherited rare mutations and single-gene disorders, including Turcot's syndrome, Li-Fraumeni syndrome, neurofibromatosis, retinoblastoma, and tuberous sclerosis, however these account for a small proportion of glioma cases [12].

Single nucleotides polymorphisms (SNPs) are one of the most common types of genetic variation in the human genome and are involved in cancer etiology and susceptibility [13]. These SNPs may occur in different regions of genes such as promoters, exons, introns and 5' and 3' untranslated regions, affecting promoter activity, transcription factors binding, microRNA binding, translational activity and/or epigenetic mechanisms [13]. SNPs in genes that regulate the cell cycle have been associated with diffuse glioma susceptibility [14–17]. Accordingly, the genetic polymorphisms most frequently studied in the context of brain tumors are located in genes that might influence susceptibility to these tumors in concert with environmental exposures, such as those involved in DNA repair, cell proliferation, apoptosis, angiogenesis or immune response [18–20].

Importantly, larger studies, including genome-wide association studies (GWAS) have identified five polymorphisms associated with risk of gliomas: rs2736100 (*TERT*); rs4295627 (*CCDC26*); rs4977756 (*CDKN2A/B*); rs6010620 (*RTEL1*); rs498872 (*PHLDB1*) in UK, USA-MDA, French, and German populations [21], European (French, German and Swedish) [22]. These SNPs are potential diagnostic and therapeutic biomarkers in different types of cancer [13].

The association of these SNPs with gliomas is currently unknown in the Portuguese population. Therefore, the aim of this study was to analyze the association of these five loci rs2736100 (*TERT*), rs4295627 (*CCDC26*), rs4977756 (*CDKN2A/B*), rs6010620 (*RTEL1*), and rs498872 (*PHLDB1*) and glioma risk in a Portuguese population.

Results

In order to validate the association of diffuse glioma susceptibility and SNPs rs4977756 (*CDKN2A/B*), rs6010620 (*RTEL1*), rs498872 (*PHLDB1*), rs2736100 (*TERT*) and rs4295627 (*CCDC26*), previously established in GWAS [21, 22], we conducted a case-control study in a Portuguese population. All studied SNPs were in Hardy-Weinberg equilibrium in the control group. More detailed information about the 127 glioma samples and 180 controls is summarized in Table 1.

Polymorphisms rs6010620 (*RTEL1*), rs4977756 (*CDKN2A/B*) and rs498872 (*PHLDB1*) are associated with glioma risk in a Portuguese population

Firstly, we conducted a univariate logistic regression analysis to validate the associations of SNPs and glioma risk in the Portuguese population (180 controls, 127 gliomas, including 87 glioblastomas) (Table 2). In this analysis, it was observed that the heterozygous AG and the homozygous GG genotypes of the rs4977756 (*CDKN2A/B*) were

Table 1 Clinical-pathological features of glioma patients

Groups (WHO grade)	Number	Age years (median $\pm$ SD)	Gender (M; F)	mOS (range)
Controls	180	35–85 (53.0 $\pm$ 10.8)	113; 67	n.a.
Gliomas (II–IV)	127	20–83 (58.0 $\pm$ 12.7)	79; 48	16.5 (1–52)
Histological subtypes of glioma				
Astrocytoma (II–IV)	102	22–83 (59.0 $\pm$ 11.9)	68; 34	16 (1–52)
Diffuse astrocytoma (II)	5	35–67 (41.0 $\pm$ 13.5)	2; 3	15.5 (9–22)
Anaplastic astrocytoma (III)	6	32–62 (47.0 $\pm$ 12.2)	3; 3	48
Glioblastoma (IV)	87	22–83 (59.5 $\pm$ 11.6)	59; 28	15.5 (1–52)
Gliosarcoma (V)	4	50–72 (61.5 $\pm$ 9.1)	4; 0	22.5 (7–31)
Oligodendroglioma (II–III)	25	20–78 (50.0 $\pm$ 14.1)	11; 14	23 (6–48)
Oligodendroglioma (II)	7	20–58 (45 $\pm$ 13.4)	2; 5	40.5 (33–48)
Anaplastic oligodendroglioma (III)	18	23–78 (53 $\pm$ 14.0)	9; 9	22 (6–48)

n.a. not applicable, mOS median overall survival (months), WHO World Health Organization

Table 2 Univariate logistic regression analysis of associations between polymorphisms and risk for glioma and glioblastoma

SNP	Control		Glioma		OR (95% CI) ^a	Glioblastoma		OR (95% CI) ^a
	N	%	N	%		N	%	
rs2736100 (<i>TERT</i> C/A)	177		124			85		
CC	50	28	44	35	–	33	39	–
CA	88	50	60	48	0.78 (0.46–1.31)	39	46	1.33 (0.41–4.24)
AA	39	22	20	16	0.58 (0.30–1.14)	13	15	2.10 (0.32–13.98)
rs6010620 (<i>RTEL1</i> G/A)	179		121			82		
GG	113	63	86	71	–	64	78	–
GA	56	31	33	27	0.77 (0.46–1.29)	18	22	0.57 (0.31–1.05)
AA	10	6	2	2	0.26 (0.06–1.23)	0	0	–
rs4295627 (<i>CCDC26</i> T/G)	180		127			87		
TT	130	72	84	66	–	62	71	–
TG	43	24	36	28	1.30 (0.77–2.18)	19	22	0.93 (0.50–1.72)
GG	7	4	7	6	1.55 (0.52–4.57)	6	7	1.80 (0.58–5.57)
rs4977756 (<i>CDKN2A/B</i> A/G)	176		118			80		
AA	82	47	34	29	–	20	25	–
AG	72	41	58	49	1.94 (1.15–3.30)	40	50	2.28 (1.22–4.25)
GG	22	13	26	22	2.85 (1.42–5.71)	20	25	3.73 (1.71–8.12)
rs498872 (<i>PHLDB1</i> G/A)	180		126			86		
GG	103	57	43	34	–	32	37	–
GA	60	33	72	57	2.87 (1.75–4.71)	44	51	2.36 (1.35–4.11)
AA	17	9	11	9	1.55 (0.67–3.58)	10	12	1.89 (0.79–4.55)

Bold-faced values indicate significant difference at the 5% level

OR (95% CI) Odds ratio with 95% confidence intervals

associated with increased risk of gliomas (AG genotype: OR 1.94, CI 1.15–3.30; GG genotype: OR 2.85, CI 1.42–5.71) and glioblastomas (AG genotype: OR 2.28, CI 1.22–4.25; GG genotype: OR 3.73, CI 1.71–8.12) (Table 2). It was also observed that the heterozygous genotype GA of the rs498872 (*PHLDB1*) was associated with an increased risk of gliomas (OR 2.87, CI 1.75–4.71) and glioblastomas (OR 2.36, CI 1.35–4.11) (Table 2). In this univariate analysis, the remained three polymorphisms rs2736100 (*TERT*),

rs6010620 (*RTEL1*), and rs4295627 (*CCDC26*) included in this study, showed no association with glioma or glioblastoma risk in the Portuguese population (Table 2).

Secondly, a multivariate logistic regression analysis adjusted for potential confounding variables such as patient's age and gender was performed. A total of 114 glioma cases and 174 controls were successfully included in the multivariate logistic analysis. The numbers and frequencies of each genotype in cases and controls are summarized in Table 3.

Table 3 Multivariate logistic regression analysis of associations between SNPs and risk for glioma and glioblastoma

SNP	Control		Glioma		OR (95% CI) ¹	p	Glioblastoma		OR (95% CI) ¹	p
	N	%	N	%			N	%		
rs2736100 (<i>TERT</i> C/A)	174		114				78			
CC	48	28	40	35	–		30	38	–	
CA	88	51	54	47	0.71 (0.39–1.29)	0.261	35	45	0.62 (0.31–1.25)	0.185
AA	38	22	20	18	0.73 (0.35–1.55)	0.414	13	17	0.68 (0.28–1.65)	0.392
rs6010620 (<i>RTEL1</i> G/A)	174		114				78			
GG	109	63	80	70	–		61	78	–	
GA	55	32	32	28	0.70 (0.39–1.24)	0.224	17	22	0.45 (0.22–0.91)	0.027
AA	10	06	2	02	0.24 (0.04–1.29)	0.096	0	0	–	–
rs4295627 (<i>CCDC26</i> T/G)	174		114				78			
TT	128	74	79	69	–		59	76	–	
TG	42	24	35	31	1.52 (0.84–2.74)	0.165	19	24	1.00 (0.48–2.10)	0.997
GG	4	02	0	0	–	–	0	0	–	–
rs4977756 (<i>CDKN2A/B</i> A/G)	174		114				78			
AA	81	47	33	29	–		20	26	–	
AG	71	41	55	48	1.85 (1.03–3.30)	0.038	38	49	2.77 (1.36–5.67)	0.005
GG	22	13	26	23	2.38 (1.13–5.04)	0.023	20	26	3.94 (1.63–9.55)	0.002
rs498872 (<i>PHLDB1</i> G/A)	174		114				78			
GG	102	0.59	42	0.37	–		31	40	–	
GA	58	0.33	67	0.59	2.92 (1.71–5.00)	<0.001	42	54	2.39 (1.26–4.53)	0.008
AA	14	0.08	5	0.04	0.73 (0.23–2.32)	0.0587	5	6	0.73 (0.21–2.60)	0.627

Bold-faced values indicate significant difference at the 5% level

OR (95% CI) Odds ratio with 95% confidence intervals

In the multivariate analysis it was observed that the heterozygous genotype AG and the homozygous GG genotype of the SNP rs4977756 (*CDKN2A/B*) were associated with an increased risk of gliomas (AG genotype: OR 1.85, CI 1.03–3.30, $p=0.038$; GG genotype: OR 2.38, CI 1.13–5.04, $p=0.023$) and glioblastomas (AG genotype: OR 2.77, CI 1.36–5.67, $p=0.005$; GG genotype: OR 3.94, CI 1.63–9.55, $p=0.002$) (Table 3). Moreover, this analysis showed that the heterozygous genotype GA of the SNP rs498872 (*PHLDB1*) was associated with an increased risk for both for gliomas (OR 2.92, CI 1.71–5.00, $p\leq 0.001$) and glioblastomas (OR 2.39, CI 1.26–4.53, $p=0.008$) (Table 3). The multivariate analysis also revealed an association between the GA heterozygous genotype of the SNP rs6010620 (*RTEL1*), with a decreased risk of glioblastoma (OR 0.45, CI 0.22–0.91, $p=0.027$) (Table 3). In this analysis, the polymorphisms rs2736100 (*TERT*) and rs4295627 (*CCDC26*) did not show significant associations with glioma risk in the Portuguese population studied (Table 3).

The false-positive report probability (FPRP) was calculated for the significant associations observed and confirmed that all the associations found in rs498872 (*PHLDB1*) and rs4977756 (*CDKN2A/B*) loci remained noteworthy (FPRP <0.5), considering the probability of association of 25% or greater (Table 4). The associations between and risk for

glioma and glioblastoma remained significant in the univariate analysis even for a prior probability of 10% in the case of glioma risk (Table 4).

SNPs rs6010620 (*RTEL1*) and rs498872 (*PHLDB1*) are associated with glioma patients' survival

We further evaluate the association of these five polymorphisms with patients' survival on Kaplan–Meier curves using the log-rank test. In these analyses, we found that the rs6010620 (*RTEL1*) and the rs498872 (*PHLDB1*) were prognostic factors (Fig. 1).

The heterozygous genotype GA of the rs6010620 (*RTEL1*) was associated with increased overall survival in patients with gliomas, when compared with the GG genotype ($p=0.044$) (Fig. 1a). It was found that the homozygous genotype AA of the rs498872 (*PHLDB1*) was associated with poorer overall survival in gliomas when compared with GA genotype ($p=0.037$), but the results were not significant when compared with GG genotype ($p=0.080$) (Fig. 1b).

The SNP rs4977756 (*CDKN2A/B*), which was associated with glioma risk (Table 3), was not associated with patients' survival (AA vs. GA $p=0.779$; AA vs. GG $p=0.739$; GA vs. GG $p=0.734$) (Fig. 1c). We did not find associations between the polymorphisms rs2736100 (*TERT*)

Table 4 False-positive report probability (FPRP) for significant associations with risk

	OR (95% CI)	Power*	Reported <i>P</i>	Prior probability			
				0.25	0.1	0.01	0.001
Univariate analysis							
Glioma risk							
rs4977756 (<i>CDKN2A/B</i> A/G)	1.94 (1.15–3.30)	0.545	0.014	0.074	0.193	0.725	0.964
	2.85 (1.42–5.71)	0.159	0.003	0.056	0.151	0.661	0.952
rs498872 (<i>PHLDB1</i> G/A)	2.87 (1.75–4.71)	0.077	<0.001	0.001	0.004	0.038	0.283
Glioblastoma risk							
rs4977756 (<i>CDKN2A/B</i> A/G)	2.28 (1.22–4.25)	0.340	0.009	0.077	0.201	0.734	0.965
	3.73 (1.71–8.12)	0.058	<0.001	0.045	0.124	0.608	0.940
rs498872 (<i>PHLDB1</i> G/A)	2.36 (1.35–4.11)	0.279	0.002	0.025	0.072	0.461	0.896
Multivariate analysis							
Glioma risk							
rs4977756 (<i>CDKN2A/B</i> A/G)	1.85 (1.03–3.30)	0.604	0.037	0.156	0.357	0.859	0.984
	2.38 (1.13–5.04)	0.325	0.024	0.178	0.394	0.878	0.986
rs498872 (<i>PHLDB1</i> G/A)	2.92 (1.71–5.00)	0.084	<0.001	0.003	0.010	0.100	0.529
Glioblastoma risk							
rs4977756 (<i>CDKN2A/B</i> A/G)	2.77 (1.36–5.67)	0.186	0.005	0.079	0.204	0.738	0.966
	3.94 (1.63–9.55)	0.067	0.002	0.098	0.245	0.781	0.973
rs498872 (<i>PHLDB1</i> G/A)	2.39 (1.26–4.53)	0.293	0.008	0.072	0.189	0.719	0.963
rs6010620 (<i>RTEL1</i> G/A)	0.45 (0.22–0.91)	0.385	0.026	0.170	0.380	0.871	0.986

Bold-faced values indicate the FPRP ≤ 0.5 for the most likely prior probability

*Estimation of statistical power to detect an OR of 2.0 with an α level equal to the observed *P* value

and rs4295627 (*CCDC26*) and the survival of patients with gliomas.

Discussion

Identification of risk factors for gliomas is important to understand tumor etiology and may contribute to cancer prevention and the development of new therapies. Besides ionizing radiation and some rare inherited syndromes known to be associated with increased risk of gliomas, the etiology of these tumors is poorly understood [23–25].

A variety of studies have demonstrated a relationship between the risk for developing glioma and the presence of genetic polymorphisms in genes involved in important cancer pathways such as neovascularization [19, 26–29] DNA repair [14]; cell differentiation/death [30]; cycle control and maintenance of telomeres [22, 27, 31]. New and important information knowledge of genetic risk factors was recently identified by genome wide association studies (GWAS) that analyzed hundreds of thousands of SNPs in a large number of cases. These GWAS have identified five important loci at at 5p15.33 rs2736100 (*TERT*); 20q13.33 rs6010620 (*RTEL1*); 8q24.21 rs4295627 (*CCDC26*); 9p21.3 rs4977756 (*CDKN2A/B*); and 11q23.3 rs498872 (*PHLDB*), associated with glioma risk [22, 27]. The present study aimed to perform a case–control study to replicate the association of

these five polymorphisms with glioma risk in a Portuguese population.

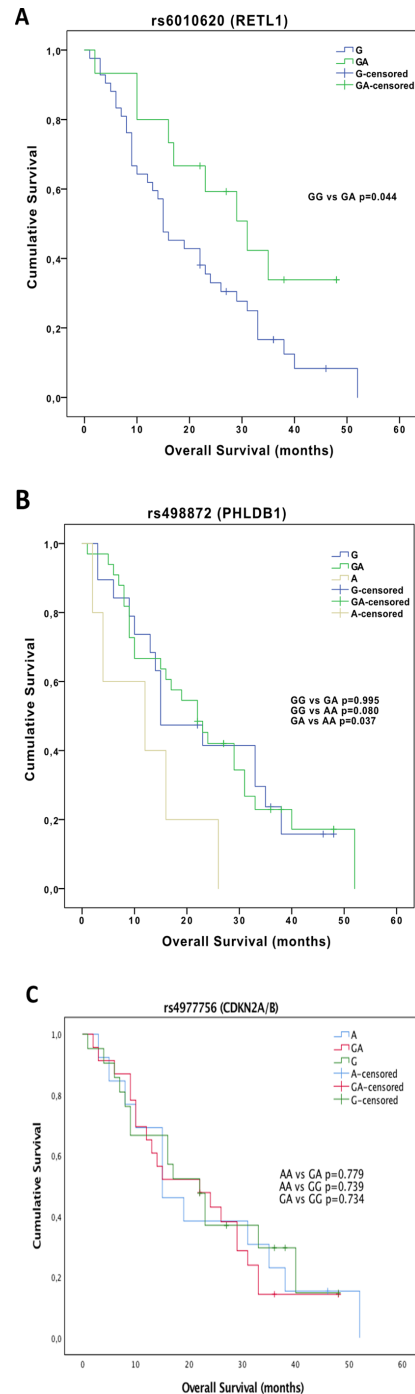
The locus rs2736100 (*TERT*) is described to be associated with an elevated risk of many cancer types, including gliomas [32, 33]. *TERT* gene encodes the catalytic subunit of the telomerase enzyme. While most somatic non-neoplastic cells have no activity or low transcriptional activity of *TERT*, this gene is frequently upregulated in cancer cells, associated with telomeres length maintenance and cell immortalization [34]. Several GWAS have demonstrated that the SNP rs2736100 (*TERT*) is associated with an increased risk of glioma [21, 22, 35, 36]. However, in the present study, we have not observed a significant association between this SNP and glioma risk. Compared to literature, different results found in the present study may be due to several issues, such as small sample size and different populations analyzed.

The locus rs6010620, reported to be associated with glioma risk, is located in the region of *RTEL1* (regulator of telomere elongation helicase 1), which is also known to be involved in telomeres' maintenance. [22, 31, 37, 38]. A meta-analysis of two GWAS, including 1878 cases and 3670 controls, with validation in 2545 cases and 2953 controls of three additional independent series, showed an association of G allele of the rs6010620 (*RTEL1*) and an increased risk of gliomas [22]. Another meta-analysis that included 12 case–control studies confirmed these results and found that the heterozygous variant GA of this SNP was associated with

Fig. 1 Survival functions of SNPs (*RET1*, *CDKN2A/B*, and *PHLDB1*) associated with glioma risk. **a** The GA variant of rs6010620 (*RET1*) is associated with higher overall survival of glioma patients compared with GG variant ($p=0.044$). **b** The homozygous variant AA of rs498872 (*PHLDB1*) is associated with poor overall survival in gliomas, compared with the GA variant ($p=0.037$). **c** SNP rs497756 (*CDKN2A/B*) showed no significant differences in glioma patients survival

a decreased risk of gliomas compared with the GG variant (OR 0.76, CI 0.71–0.81) in Asian and Caucasian ethnicities [31]. Corroborating the previous studies, we also observed that the heterozygous variant GA of the SNP rs6010620 was associated with a decreased risk of glioblastoma (OR 0.45, CI 0.22–0.91, $p=0.027$), confirmed by FPRP calculation with a probability of association of 25%. We also observed that the GA variant of the SNP rs6010620 was associated with higher overall survival in glioma patients suggesting that the occurrence of this variant represents a protector factor against gliomas in the Portuguese population. According to studies of integrated information derived from GWAS (FunciSNP) [39], this SNP seems to be functionally relevant in brain tumors. Hypothetically, polymorphisms in *RET1* may alter the binding proprieties of transcription factors, causing a dysfunction on telomeres elongation and therefore compromising the glioma cells' survival. Nevertheless, molecular experiments are necessary to elucidate the biological function of this SNP and to confirm this hypothesis.

The third SNP that we aimed to validate associations with glioma risk in the Portuguese population was the SNP rs4295627, located in the regions of the gene *CCDC26* (coiled-coil domain containing 26). This gene encodes a long noncoding RNA that acts as a retinoic modulator of myeloid differentiation and death, possibly associated with cancer development [40]. Meta-analyses studies showed that the SNP rs4295627 had been associated with glioma risk in Northern European [41], German [21, 22], French, and Swedish populations [22], but there was evidence of between-study heterogeneity due to the weaker association in the Swedish series [22]. Interestingly, a meta-analysis of 14 case–control studies, including 17,419 cases and 28,465 controls, showed different associations of rs4295627 in Caucasian and Asian populations. The SNP rs4295627 was associated with an increased risk for developing gliomas in Caucasians but with a decreased risk for gliomas in Asians [30]. In the present study, we did not observe an association between the SNP rs4295627 and glioma risk in the Portuguese population, suggesting that the association of this SNP with glioma risk is highly dependent on the population analyzed. Interestingly, previous reports found significant associations between the SNP rs4295627 (*CCDC26*) and increased risk for oligodendroglioma, but not with glioblastoma [30]. Genetic variants on region 8q24.21 of *CCDC26* gene were also reported to be associated with the risk of



oligodendroglial tumors and astrocytomas harboring *IDH1* or *IDH2* mutation [42]. Further studies are needed to elucidate the relationship between the SNP rs4295627 and the increased risk of *IDH* mutated oligodendrogliomas.

The fourth SNP studied in the present work was the rs4977756 that encompassed a common region of *CDKN2A* and *CDKN2B* tumor suppressor genes and was also described to be associated with an elevated risk of gliomas. The *CDKN2A* gene encodes two proteins, the P16 (INK4A) that acts as a cyclin-dependent kinases inhibitor and the P14 (ARF1), an activator of P53. The *CDKN2B* encodes P15, another cyclin-dependent kinase inhibitor [22, 43–45]. A meta-analysis of 12 case–control studies, including a total of 12,022 controls and 6871 cases, demonstrated that the variant G allele of the SNP rs4977756 (*CDKN2A/B*) was associated with an increased risk for glioma in Europeans [44]. GWAS had also described similar results, with heterozygote (GA) and homozygote (GG) genotypes associated with an increased risk of gliomas [22]. Our findings corroborate with these results regarding the heterozygous and homozygous genotypes, with a probability of association of 25% or more (FPRP) in the Portuguese population studied. An additional meta-analysis of 13 case–control studies, also described that the presence of the G allele in the SNP rs4977756, was associated with an increased risk of gliomas, including Caucasian and Asian patients. Yet, the subgroup analyses of ethnicities have not confirmed this association in Asians alone [43], stressing the influence of ethnicity on polymorphisms studies.

Finally, we have studied the association between the SNP rs498872 (*PHLDB1*) and glioma risk. The molecular and biological functions of the transcription factor PHLDB1 (Pleckstrin Homology-Like Domain Family B member 1), remains poorly understood, but it seems to be involved in cancer pathways like cell proliferation, apoptosis, differentiation and migration [46]. GWAS have identified that the T allele of the SNP rs498872 (*PHLDB1*) is associated with an increased risk of gliomas [22]. These results are in accordance with another meta-analysis, including 10 case–control studies with 18,002 controls and 8434 cases, which described that homozygous TT, heterozygous TC variants of the SNP rs498872 were associated with an increased risk of gliomas [47]. In the present study, we observed that the heterozygous variant GA of the SNP rs498872 (*PHLDB1*) was associated with an increased risk of gliomas (OR 2.92, CI 1.71–5.00, $p < 0.001$) and glioblastomas (OR 2.39, CI 1.26–4.53, $p = 0.008$) in the Portuguese population studied. The analysis of FPRP showed that the probability of association was 1% or greater for glioma risk and above 10% for glioblastoma risk. Also, the homozygous variant AA of the rs498872 (*PHLDB1*) was associated with poor overall survival in gliomas. The small sample size of this study is probably a relevant factor that contributes to these different

findings in relation to the SNP rs498872, and therefore the conduction of studies with a larger number of samples should be conducted to confirm these results.

Besides commonly referred as a uniform genetic ancestry, the European populations are heterogeneous. The genotyping of 500,568 loci in 3,192 European individuals found a close correlation between genetic and geographic distances [48]. Geographically adjacent populations commonly confine themselves and consequently have more uniform ancestry. With a few exceptions of recent migrant small populations, the genetic correlation data between individuals tends to decay with distance and geographic distribution is important to consider in GWAS among Europeans [48].

In this context, the Iberian populations (Portuguese and Spanish), have a more uniform SNP background compared to the other Europeans populations [48]. This may be related to some different results observed in the present study when compared to other GWAS on polymorphisms and glioma risk in different European populations.

Conclusion

In the present study, we showed that the polymorphisms rs6010620 (*RTEL1*), rs4977756 (*CDKN2A/B*), and rs498872 (*PHLDB1*) were associated with glioma risk, corroborating previous GWAS reports [21, 22]. Moreover, polymorphisms rs6010620 (*RTEL1*) and rs498872 (*PHLDB1*) are associated with gliomas patients' survival in the Portuguese population studied. These findings may contribute to the understanding of gliomas risk factors.

Methods

Samples

This retrospective case–control study, enrolled a cohort of 127 diffuse glioma samples, including 40 lower-grade gliomas (WHO grades II and III) and 87 glioblastomas (grade IV) from Hospital São João, Porto, and Hospital of Braga, Braga, Portugal as previously described [29] (Table 1). The median age of glioma patients was 58.0 years (SD = 12.7), most was from the male gender (79/48), and the median overall survival was 16.5 months, ranging from 1 to 52 months (Table 1). It was also included 180 controls cancer-free samples of blood donors from Braga Hospital (Table 1). Patients and controls included in this study have a Caucasian background and are from the north of Portugal. This study was approved for the institutional ethics committees of São João and Braga Hospitals (Approval 29/10/2007).

Genotyping

DNA was extracted from blood samples using the Citogene® Blood Kit (Citomed, Lisbon, Portugal) according to the manufacturer's instructions. Genotyping was performed using the Sequenom MassARRAY iPLEX Gold platform (Sequenom, San Diego, California) at the Instituto Gulbenkian de Ciência, Lisbon, Portugal, and quality were assessed by duplicate analysis of 10% of the samples, which demonstrated a 100% agreement rate. Primers for the loci of interest were designed using MassARRAY Assay Design 3.1 software (Sequenom, San Diego, California).

Statistical analysis

Data analysis was performed using IBM® SPSS Statistics, version 25.0. A Chi squared test was used to confirm whether the observed allele distribution in the control group was in Hardy–Weinberg equilibrium. The effect of the polymorphic variants on the risk for glioma was estimated using univariate or multivariate logistic regression analysis, this last one, adjusted for gender and age in terms of odds ratio (OR) and 95% confidence intervals (95% CI). Analysis of overall survival (OS) was performed using the Kaplan–Meier method and the log-rank test. For significant associations observed in multivariate tests, the false-positive report probability (FPRP) was calculated [49]. All statistical tests were two-sided, and significance was considered for $P < 0.05$.

Author contributions MV-P: Genotyping of samples, analysis of polymorphisms and patient's data, tables and figures, manuscript review; DAM: interpreted genotyping, patient's data, bibliography review, manuscript writing; PL, JA, RN and RV: clinical data collection, interpretation and final review; SC: collections of samples, DNA extraction, genotyping; RMR: study design, scientific support, results analysis, manuscript review, research coordination.

Funding The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was funded by Fundação para a Ciência e Tecnologia (FCT), Portugal (www.fct.pt; grant PTDC/SAU-ONC/115513/2009_FCOMP-01-0124-FEDER-015949 to R.M.R.). This article has been developed under the scope of the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER). M.V.P. was recipient of an FCT Post-Doctoral fellowship (ref: SFRH/BPD/104290/2014).

Data availability Samples and clinical data of participants are available in São João and Porto Hospitals of Braga, Portugal.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Institutional ethics committees of S. João and Braga Hospitals (Approval 29/10/2007).

Informed consent Not applicable. This is a retrospective study where any information that allows participants identification is not disclosed.

References

- Alcantara Llaguno SR, Parada LF (2016) Cell of origin of glioma: biological and clinical implications. *Br J Cancer* 115(12):1445–1450. <https://doi.org/10.1038/bjc.2016.354>
- Ohgaki H, Kleihues P (2005) Epidemiology and etiology of gliomas. *Acta Neuropathol* 109(1):93–108. <https://doi.org/10.1007/s00401-005-0991-y>
- Paolillo M, Boselli C, Schinelli S (2018) Glioblastoma under Siege: an overview of current therapeutic strategies. *Brain Sci.* <https://doi.org/10.3390/brainsci8010015>
- Wen PY, Reardon DA (2016) Neuro-oncology in 2015: progress in glioma diagnosis, classification and treatment. *Nat Rev Neurol* 12(2):69–70. <https://doi.org/10.1038/nrneurol.2015.242>
- Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK et al (2016) The 2016 World Health Organization classification of tumors. *Acta Neuropathol* 131(6):803–820. <https://doi.org/10.1007/s00401-016-1545-1>
- Batista R, Cruvinel-Carlioni A, Vinagre J, Peixoto J, Catarino TA, Campanella NC et al (2016) The prognostic impact of TERT promoter mutations in glioblastomas is modified by the rs2853669 single nucleotide polymorphism. *Int J Cancer* 139(2):414–423. <https://doi.org/10.1002/ijc.30057>
- Eckel-Passow JE, Lachance DH, Molinaro AM, Walsh KM, Decker PA, Sicotte H et al (2015) Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *N Engl J Med* 372:2499–2508. <https://doi.org/10.1056/NEJMoa1407279>
- Wesseling P, Capper D (2018) WHO 2016 classification of gliomas. *Neuropathol Appl Neurobiol* 44(2):139–150. <https://doi.org/10.1111/nan.12432>
- Brat DJ, Verhaak RG, Aldape KD, Yung WK, Salama SR, Cooper LA et al (2015) Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *N Engl J Med* 372(26):2481–2498. <https://doi.org/10.1056/NEJMoa1402121>
- Noroxe DS, Poulsen HS, Lassen U (2016) Hallmarks of glioblastoma: a systematic review. *ESMO Open* 1(6):e000144. <https://doi.org/10.1136/esmoopen-2016-000144>
- Ohgaki H (2009) Epidemiology of brain tumors. *Methods Mol Biol* 472:323–342. https://doi.org/10.1007/978-1-60327-492-0_14
- Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M (2006) Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol* 2(9):494–503. <https://doi.org/10.1038/ncpneuro0289>
- Deng N, Zhou H, Fan H, Yuan Y (2017) Single nucleotide polymorphisms and cancer susceptibility. *Oncotarget* 8(66):110635–110649. <https://doi.org/10.18632/oncotarget.22372>
- Adel Fahmideh M, Schwartzbaum J, Frumeto P, Feyshting M (2014) Association between DNA repair gene polymorphisms and risk of glioma: a systematic review and meta-analysis. *Neuro Oncology* 6(6):807–814. <https://doi.org/10.1093/neuonc/nou003>
- Liu Y, Scheurer ME, El-Zein R, Cao Y, Do KA, Gilbert M et al (2009) Association and interactions between DNA repair gene polymorphisms and adult glioma. *Cancer Epidemiol*

- Biomarkers Prev 18(1):204–214. <https://doi.org/10.1158/1055-9965.EPI-08-0632>
16. McKean-Cowdin R, Barnholtz-Sloan J, Inskip PD, Ruder AM, Butler M, Rajaraman P et al (2009) Associations between polymorphisms in DNA repair genes and glioblastoma. *Cancer Epidemiol Biomarkers Prev* 18(4):1118–1126. <https://doi.org/10.1158/1055-9965.EPI-08-1078>
 17. Zawlik I, Kita D, Vaccarella S, Mittelbronn M, Franceschi S, Ohgaki H (2009) Common polymorphisms in the MDM2 and TP53 genes and the relationship between TP53 mutations and patient outcomes in glioblastomas. *Brain Pathol* 19(2):188–194. <https://doi.org/10.1111/j.1750-3639.2008.00170.x>
 18. Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A et al (2007) Association between functional EGF + 61 polymorphism and glioma risk. *Clin Cancer Res* 13(9):2621–2626. <https://doi.org/10.1158/1078-0432.CCR-06-2606>
 19. Costa BM, Viana-Pereira M, Fernandes R, Costa S, Linhares P, Vaz R et al (2011) Impact of EGFR genetic variants on glioma risk and patient outcome. *Cancer Epidemiol Biomarkers Prev* 20(12):2610–2617. <https://doi.org/10.1158/1055-9965.EPI-11-0340>
 20. Pandey JP, Kaur N, Costa S, Amorim J, Nabico R, Linhares P et al (2014) Immunoglobulin genes implicated in glioma risk. *Oncoimmunology* 3:e28609. <https://doi.org/10.4161/onci.28609>
 21. Rajaraman P, Melin BS, Wang Z, McKean-Cowdin R, Michaud DS, Wang SS et al (2012) Genome-wide association study of glioma and meta-analysis. *Hum Genet* 131(12):1877–1888. <https://doi.org/10.1007/s00439-012-1212-0>
 22. Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B et al (2009) Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet* 41(8):899–904. <https://doi.org/10.1007/s00439-012-1212-0>
 23. Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D et al (2008) Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. *Cancer* 113(7 Suppl):1953–1968. <https://doi.org/10.1002/cncr.23741>
 24. Melin B, Jenkins R (2013) Genetics in glioma- lessons learned from genome wide association studies. *Curr Opin Neurol* 26(6):688–692. <https://doi.org/10.1097/WCO.0000000000000033>
 25. Ostrom QT, Gittleman H, Stetson L, Virk S, Barnholtz-Sloan JS (2018) Epidemiology of intracranial gliomas. *Prog Neurol Surg* 30:1–11. <https://doi.org/10.1159/000464374>
 26. Chen X, Yang G, Zhang D, Zhang W, Zou H, Zhao H et al (2014) Association between the epidermal growth factor +61 G/A polymorphism and glioma risk: a meta-analysis. *PLoS ONE* 9(4):e95139. <https://doi.org/10.1371/journal.pone.0095139>
 27. Ghasimi S, Wibom C, Dahlin AM, Brännström T, Golovleva I, Andersson U et al (2016) Genetic risk variants in the CDKN2A/B, RTEL1 and EGFR genes are associated with somatic biomarkers in glioma. *J Neurooncol* 127(3):483–492. <https://doi.org/10.1007/s11060-016-2066-4>
 28. Sanson M, Hosking FJ, Shete S, Zelenika D, Dobbins SE, Ma Y (2011) Chromosome 7p11.2 (EGFR) variation influences glioma risk. *Hum Mol Genet* 20(14):2897–2904. <https://doi.org/10.1093/hmg/ddr192>
 29. Linhares P, Viana-Pereira M, Ferreira M, Amorim J, Nabico R, Pinto F et al (2018) Genetic variants of vascular endothelial growth factor predict risk and survival of gliomas. *Tumor Biol* 40(3):1–11. <https://doi.org/10.1177/1010428318766273>
 30. Cui T (2015) CCDC26 rs4295627 polymorphism and glioma risk: a meta-analysis. *Int J Clin Exp Med* 8(3):3862–3868
 31. Du SL, Geng TT, Feng T, Chen CP, Jin TB, Chen C (2014) The RTEL1 rs6010620 polymorphism and glioma risk: a meta-analysis based on 12 case-control studies. *Asian Pac J Cancer Prev* 15(23):10175–10179. <https://doi.org/10.7314/apjcp.2014.15.23.10175>
 32. Li H, Xu Y, Mei H, Peng L, Li X, Tang J (2017) The TERT rs2736100 polymorphism increases cancer risk: a meta-analysis. *Oncotarget* 8(24):38693–38705. <https://doi.org/10.18632/oncotarget.16309>
 33. Walsh KM, Codd V, Smirnov IV, Rice T, Decker PA, Hansen HM et al (2014) Variants near TERT and TERC influencing telomere length are associated with high-grade glioma risk. *Nat Genet* 46(7):731–735. <https://doi.org/10.1038/ng.3004>
 34. Pestana A, Vinagre J, Sobrinho-Simões M, Soares P (2017) TERT biology and function in cancer: beyond immortalisation. *J Mol Endocrinol* 58(2):R129–R146. <https://doi.org/10.1530/JME-16-0195>
 35. Wrensch M, Jenkins RB, Chang JS, Yeh RF, Xiao Y, Decker PA et al (2009) Variants in the CDKN2B and RTEL1 regions are associated with high-grade glioma susceptibility. *Nat Genet* 41(8):905–908. <https://doi.org/10.1038/ng.408>
 36. Schoemaker MJ, Robertson L, Wigertz A, Jones ME, Hosking FJ, Feychting M et al (2010) Interaction between 5 genetic variants and allergy in glioma risk. *Am J Epidemiol* 171:1165–1173. <https://doi.org/10.1093/aje/kwq075>
 37. Wu Y, Tong X, Tang LL, Zhou K, Zhong CH, Jiang S (2014) Associations between the rs6010620 polymorphism in RTEL1 and risk of glioma: a meta-analysis of 20,711 participants. *Asian Pac J Cancer Prev* 15(17):7163–7167. <https://doi.org/10.7314/apjcp.2014.15.17.7163>
 38. Zhao W, Bian Y, Zhu W, Zou P, Tang G (2014) Regulator of telomere elongation helicase 1 (RTEL1) rs6010620 polymorphism contribute to increased risk of glioma. *Tumour Biol* 35(6):5259–5266. <https://doi.org/10.1007/s13277-014-1684-8>
 39. Coetzee SG, Rhie SK, Berman BP, Coetzee GA, Noushmehr H (2012) FunciSNP: an R/bioconductor tool integrating functional non-coding data sets with genetic association studies to identify candidate regulatory SNPs. *Nucleic Acids Res* 40(18):e139. <https://doi.org/10.1093/nar/gks542>
 40. Hirano T (2016) The role of the CCDC26 long noncoding RNA as a tumor suppressor. *RNA Dis* 3:e1022
 41. Enciso-Mora V, Hosking FJ, Kinnersley B, Wang Y, Shete S, Zelenika D et al (2013) Deciphering the 8q24.21 associations for glioma. *Hum Mol Genet* 22(11):2293–2302
 42. Jenkins RB, Wrensch MR, Johnson D, Fridley BL, Decker PA, Xiao Y et al (2011) Distinct germ line polymorphisms underlie glioma morphologic heterogeneity. *Cancer Genet* 204(1):13–18. <https://doi.org/10.1016/j.cancergencyto.2010.10.002>
 43. Qi X, Wan Y, Zhan Q, Yang S, Wang Y, Cai X (2016) Effect of CDKN2A/B rs4977756 polymorphism on glioma risk: a meta-analysis of 16 studies including 24077 participants. *Mamm Genome* 27(1–2):1–7. <https://doi.org/10.1007/s00335-015-9612-9>
 44. Lu H, Yang Y, Wang J, Liu Y, Huang M, Sun X et al (2015) The CDKN2A-CDKN2B rs4977756 polymorphism and glioma risk: a meta-analysis. *Int J Clin Exp Med* 8(10):17480–17488
 45. Yang TH, Kon M, Hung JH, Delisi C (2011) Combinations of newly confirmed glioma-associated loci link regions on chromosomes 1 and 9 to increased disease risk. *BMC Med Genomics* 4:63. <https://doi.org/10.1186/1755-8794-4-63>
 46. Nagai MA (2016) Pleckstrin homology-like domain, family A, member 1 (PHLDA1) and cancer. *Biomed Rep* 4(3):275–281. <https://doi.org/10.3892/br.2016.580>
 47. Gao X, Mi Y, Yan A, Sha B, Guo N, Hu Z et al (2015) The PHLDB1 rs498872 (11q23.3) polymorphism and glioma risk: a meta-analysis. *Asia Pac J Clin Oncol* 11(4):e13–e21. <https://doi.org/10.1111/ajco.12211>
 48. Novembre J, Johnson T, Bryc K, Kutalik Z, Boyko AR, Auton A, Indap A, King KS, Bergmann S, Nelson MR, Stephens M,

- Bustamante CD (2008) Genes mirror geography within Europe. *Nature* 456(7218):98–101. <https://doi.org/10.1038/nature07331>
49. Wacholder S, Chanock S, Garcia-Closas M, El GL, Rothman N (2004) Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 96:434–442. <https://doi.org/10.1093/jnci/djh075>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

8 BODY MASS INDEX AS AN INDEPENDENT PROGNOSTIC FACTOR IN GLIOBLASTOMA

Pedro Valente Aguiar,^{1,2} Bruno Carvalho,^{1,2} Rui Vaz,^{1,2,3} Paulo Linhares,^{1,2,3}

¹ Neurosurgery Department, Centro Hospitalar Universitário São João, Porto, Portugal; ² Faculty of Medicine, Porto University, Porto, Portugal; ³ Neurosciences Centre, Hospital da CUF, Porto, Portugal.



Body mass index as an independent prognostic factor in glioblastoma

Pedro Valente Aguiar^{1,2} · Bruno Carvalho^{1,2} · Rui Vaz^{1,2,3} · Paulo Linhares^{1,2,3}

Received: 11 August 2020 / Accepted: 22 December 2020

© The Author(s), under exclusive licence to Springer Nature Switzerland AG part of Springer Nature 2021

Abstract

Purpose Glioblastoma prognosis remains dismal despite gross total removal (GTR) followed by chemoradiotherapy. Other known prognostic factors include functional status, age and IDH mutation status. However, to improve patient outcome, a search for other features with impact on survival is needed. We aimed to analyse the impact of body mass index (BMI) on overall survival (OS) and progression-free survival (PFS) of surgically resected primary glioblastoma and evaluate if BMI constitutes an independent prognostic factor.

Methods We analysed all adult glioblastoma patients who underwent surgery and chemoradiotherapy between 2011 and 2017 at our institution. Overall survival was the study—primary endpoint, and progression-free survival—the secondary endpoint. We assayed age, gender, histology, extent of resection, IDH, functional and smoking status, cardiovascular risk factors, BMI, OS and PFS. Univariate analysis was conducted followed by multivariate analysis to establish independent prognostic factors. In accordance with the World Health Organization (WHO) BMI stratification, survival curves were obtained for normal-weight (18.5–24.9 kg/m²), overweight (25–29.9 kg/m²) and obese (≥ 30 kg/m²) patient subgroups in addition to the non-obese (18.5–29.9 kg/m²) population.

Results 193 patients were evaluated, with a median follow-up time of 17.3 months. Median OS was 21.3 months in obese patients vs 16.2 months in the non-obese ($p=0.017$) and 16 months in the normal weight ($p=0.007$). Higher median OS was also observed in patients under 60 and those in which GTR was obtained. Median PFS in obese individuals was 9 months in comparison to 6 months in the normal-weight subgroup ($p=0.04$) and 7 months in the non-obese ($p=0.050$). Multivariate analysis identified age < 60 ($p=0.044$), GTR ($p=0.004$) and BMI ≥ 30 ($p=0.009$) as independent prognostic factors for increased overall survival.

Conclusion Higher BMI was associated with longer OS and PFS. Prospective studies are needed to validate these findings.

Keywords Glioblastoma · Body mass index · Obese · Overall survival · Progression-free survival

Abbreviations

GBM	Glioblastoma	OS	Overall survival
RT	Radiotherapy	PFS	Progression-free survival
TMZ	Temozolomide	CHUSJ	Centro Hospitalar Universitário São João
TTFs	Tumour-treating fields	WHO	World Health Organization
GTR	Gross total removal	MGMT	O-6-Methylguanine-DNA methyltransferase
IDH	Isocitrate dehydrogenase	HGG	Higher-grade glioma
CNS	Central nervous system	RCC	Renal cell cancer
BMI	Body mass index	CSS	Cancer-specific survival
EOR	Extent of resection	FASN	Fatty acid synthase

✉ Pedro Valente Aguiar
pedrovalenteaguiar@protonmail.com

¹ Neurosurgery Department, Centro Hospitalar Universitário São João, Porto, Portugal

² Faculty of Medicine, Porto University, Porto, Portugal

³ Neurosciences Centre, Hospital da CUF, Porto, Portugal

Introduction

Glioblastoma (GBM) is the most aggressive primary malignant brain tumour in the adult population. Despite increased survival after the introduction of radiotherapy (RT), temozolomide (TMZ) and more recently tumour-treating fields

Published online: 15 January 2021

Springer

(TTFields), to the therapeutic plan, prognosis remains dire with 5-year survival under 5% [1].

Gross total removal (GTR) followed by chemoradiotherapy is associated with better outcomes [2]. Age, functional performance status and isocitrate dehydrogenase (IDH) mutation status are also important prognostic factors [1].

Excess body weight and obesity are known risk factors for the development of various neoplasms, and in the central nervous system (CNS), meningiomas have been linked to higher body weight [3–6]. Hormonal imbalance, chronic inflammation and increased insulin resistance are among the factors associated with increased cancer risk in overweight and obese patients [7, 8]. Furthermore, in some studies, excess body weight had a negative impact on cancer morbidity and mortality [9, 10].

The association between body mass index (BMI) and glioma occurrence is less clear, and no association has thus far been found between higher body weight and glioma risk [11–13].

In addition, there is conflicting evidence regarding BMI impact on higher-grade glioma patients' survival [14–17].

In 2013, Siegel et al. reported decreased survival of pre-diagnostic underweight and obese higher-grade glioma subjects [14]. However, BMI was subjectively assayed based on patients' own perceived weight 1–5 years prior to diagnosis, and multivariate analysis was not adjusted for extent of resection (EOR).

In a prospective study published in 2010, Jones and colleagues found no association between body weight and survival rates in 1,259 previously untreated glioblastoma patients diagnosed between 1991 and 2008 [15].

However, due to the study's timeframe, most patients did not undergo chemoradiotherapy according to the Stupp protocol, which has become the standard of care in glioblastoma treatment [16].

More recently, Potharaju et al. reported increased survival of overweight and obese GBM patients [17].

In this study, we aimed to evaluate the impact of BMI on overall survival (OS) and progression-free survival (PFS) of surgically resected primary glioblastoma and to assess whether BMI constitutes an independent prognostic factor.

Methods

Study design and patient selection

We retrospectively reviewed the clinical files of all adult glioblastoma patients who underwent surgery and adjuvant chemoradiotherapy treatment between 2011 and 2017 at Centro Hospitalar Universitário São João (CHUSJ), Porto, Portugal.

A total of 193 patients with histologically proven GBM were evaluated for clinical progression and outcome through assessment of medical records from Neurosurgery, Oncology and Radiotherapy inpatient and outpatient consultations.

Patients with post-op vascular lesions or systemic infections that precluded chemoradiation, secondary glioblastomas and biopsies were excluded. Additionally, one underweight, two normal-weight and one overweight patient were not included due to aggressive tumour recurrence before initiating or completing the first cycle from the Stupp protocol. We evaluated extent of resection, age, gender, functional status (ECOG), tumour histology, BMI, IDH-status, PFS and OS. Additionally, smoking status and cardiovascular risk factors including hypertension, dyslipidaemia and diabetes mellitus were assayed.

Overall survival was calculated from date of surgery to date of death or when alive to last medical evaluation. Progression-free survival was calculated from date of surgery to date of imaging or clinical recurrence, whatever took place first, or when progression did not occur to last medical consultation.

BMI was obtained from oncology outpatient consultations before the start of chemoradiotherapy.

Body mass index was classified according to World Health Organization (WHO) categories: normal (18.5–24.9 kg/m²), overweight (25–29.9 kg/m²) and obese (≥ 30 kg/m²).

Besides comparison within WHO BMI subsets, we further compared overall survival and progression-free survival of obese patients with normal-weight plus overweight individuals.

Glioblastoma diagnosis was obtained by histological analysis of surgical specimens by the Department of Pathology at our institution while IDH 1/2 mutation status was confirmed through immunohistochemistry and genotyping.

189 patients had de novo IDH-wild-type glioblastoma while 4 patients had de novo IDH—mutant glioblastoma.

O-6-Methylguanine-DNA methyltransferase (MGMT) gene promoter methylation status was not routinely performed at our institution until 2017, and thus, results were only obtained for four patients.

192 patients were submitted to chemoradiation according to the Stupp protocol while a normal-weight elderly patient with positive MGMT promoter methylation status completed treatment with temozolomide. Second-line therapy after progression was bevacizumab-based in all subjects, and 26 patients had one surgery for tumour recurrence while two patients had two re-operations.

The diagnosis of hypertension, dyslipidaemia and diabetes mellitus had previously been established by the patients' general practitioners or by previous hospitalizations and was obtained through the patients' medical records.

Data and statistical analysis

We used IBM SPSS Statistics 26 software.

Overall survival was the study—primary endpoint, and progression-free survival—the secondary endpoint. A *p* value below 0.05 was considered statistically significant. Differences between groups were evaluated with Chi-square or Mann–Whitney *U* tests.

We performed univariate analysis of BMI, gender, smoking status, cardiovascular risk factors and known prognostic factors including age, functional status and EOR.

Current smokers and ex-heavy smokers (≥ 1 pack of cigarettes per day in the previous 15 years) were considered as smokers.

For univariate analysis, a post-op residual tumour volume $< 2.5 \text{ cm}^3$ and $< 2\%$ on MRI was considered GTR while age-wise patients were divided into two groups: < 60 and ≥ 60 years.

For performance status, patients were divided into two groups: ECOG 0–1 and ECOG 2–4.

We conducted multivariate analysis of OS and PFS through Cox proportional hazards regression for variables with *p* value < 0.10 on univariate analysis in order to determine independent prognostic factors.

PFS and OS curves were obtained through the Kaplan–Meier method with 95% confidence intervals for the three categories of BMI and the normal plus overweight subgroup.

Results

A total of 193 patients were evaluated, 116 men (60.1%) and 77 women (39.9%), with a median age of 60 years. 32 patients were obese, 102 were overweight, while the remaining 59 had normal weight.

Median overall survival and follow-up time for all 193 patients was 17.3 months while median progression-free survival was 7.5 months.

At the time of last medical follow-up, eight (25%) obese, 13 (12.7%) overweight and two (3.4%) normal-weight patients were alive while two (6.3%) obese and six (5.9%) overweight subjects had not shown signs of imaging or clinical tumour recurrence. In all normal-weight patients, disease progression had occurred.

Patient groups

Table 1 details patients' characteristics by BMI group.

Chi-square and Mann–Whitney *U* tests were run to rule out discrepancies within groups for age, EOR, performance and smoking status.

Median age was 62 years for obese subjects, 57 years for the non-obese (Mann–Whitney *U* test $p = 0.234$), 60 years for overweight patients ($p = 0.335$) and 59 years for the normal weight ($p = 0.495$).

Similarly, between the aforementioned groups, no statistical difference was found in ECOG performance status, smoking status and EOR.

Univariate analysis

Table 2 details *p* values obtained on univariate analysis.

Median overall survival was 21.3 months (CI 16.9–25.7) in subjects with BMI $\geq 30 \text{ kg/m}^2$ and 16.2 months in the non-obese (CI 14–18.3) (p value = 0.017).

Median OS was 19.3 months (CI 17–21.6) in those aged < 60 and 14.8 months in patients aged ≥ 60 (CI 13.2–16.4) (p value = 0.020).

Patients with GTR had a median OS of 19 months (CI 15.8–22.2) that compared to 14.6 months in subtotal resection (CI 11.1–18) (p value = 0.001).

Median OS was 18 months for ECOG 0–1 patients (CI 15.9–20) and 14.6 months in the ECOG 2–4 subgroup (CI 11.5–17.7) (p value = 0.085).

Median OS was 18.1 months (CI 15.2–21) and 17 months (CI 15–18.9) for females and males, respectively (p -value = 0.127).

Median OS was 15.2 months (CI 10.7–19.7) and 18 months (CI 15.8–20.2) for diabetic and non-diabetic subjects, respectively ($p = 0.099$).

Median progression-free survival was 9 months (CI 7.4–10.6) and 7 months (CI 6.3–7.7) in the obese and the non-obese patient subgroups, respectively ($p = 0.050$).

Patients with GTR had a median PFS of 8 months (CI 7.1–8.8) while those with subtotal resection had a median PFS of 6 months (CI 5–7) ($p = 0.037$).

ECOG, cardiovascular risk factors, smoking status, age and gender had no statistically significant impact on PFS.

Median OS and PFS within groups

Tables 3 and 4 detail OS and PFS within BMI subgroups and within gender.

Figure 1 includes survival curves for age, EOR, OS and PFS according to BMI category.

Median OS was 21.3 months in the obese subgroup (CI 16.9–25.7), 17.2 months (CI 13.5–20.9) in overweight patients ($p = 0.184$) and 16 months (CI 13.9–18) in the normal-weight population ($p = 0.007$).

Median PFS was 9 months in the obese subgroup (CI 7.4–10.6), 7 months (CI 5.8–8.2) in overweight patients ($p = 0.178$) and 6 months (CI 4.9–7) in the normal-weight population ($p = 0.004$).

Table 1 Patient characteristics by BMI category

BMI category	Normal	Overweight	Obese
Total patients (<i>n</i> = 193)	59 (30.6%)	102 (52.8%)	32 (16.6%)
Males (<i>n</i> = 116) (60.1%)	36 (61.1%)	69 (67.6%)	11 (34.4%)
Females (<i>n</i> = 77) (39.9%)	23 (38.9%)	33 (32.4%)	21 (65.6%)
Extent of resection			
Total (<i>n</i> = 111) (57.5%)	29 (49.1%)	60 (58.8%)	22 (68.8%)
Subtotal (<i>n</i> = 82) (42.5%)	30 (50.9%)	42 (41.2%)	10 (31.2%)
ECOG performance status			
ECOG 0–1 (<i>n</i> = 156) (80.8%)	47 (79.7%)	83 (76.1%)	26 (81.2%)
ECOG 2–4 (<i>n</i> = 37) (19.2%)	12 (20.3%)	19 (23.9%)	6 (18.8%)
Age	Median age = 59 years	Median age = 60 years	Median age = 62 years
< 60 years (<i>n</i> = 98) (50.7%)	30 (50.9%)	50 (49%)	18 (56.2%)
≥ 60 years (<i>n</i> = 95) (49.3%)	29 (49.1%)	52 (51%)	14 (43.8%)
IDH status			
Wild type (<i>n</i> = 189) (98.5%)	58 (98.3%)	100 (98%)	31 (96.9%)
IDH mutated (<i>n</i> = 4) (1.5%)	1 (1.7%)	2 (2%)	1 (3.1%)
Smoking status			
Smoker (<i>n</i> = 28) (14.5%)	8 (13.6%)	16 (15.7%)	4 (12.5%)
Non-smoker (<i>n</i> = 165) (85.5%)	51 (86.4%)	86 (84.3%)	28 (87.5%)
Diabetes mellitus			
Yes (<i>n</i> = 28) (14.5%)	5 (8.5%)	15 (14.7%)	8 (25%)
No (<i>n</i> = 165) (85.5%)	54 (91.5%)	87 (85.3%)	24 (75%)
Hypertension			
Yes (<i>n</i> = 81) (41.9%)	14 (23.7%)	45 (44.1%)	22 (68.8%)
No (<i>n</i> = 112) (58.1%)	45 (76.3%)	57 (55.9%)	10 (31.2%)
Surgery for recurrence			
0 (<i>n</i> = 165) (85.5%)	50 (84.7%)	87 (85.3%)	28 (87.5%)
1 (<i>n</i> = 26) (13.5%)	8 (13.6%)	14 (13.8%)	4 (12.5%)
2 (<i>n</i> = 2) (1%)	1 (1.7%)	1 (0.9%)	0
Median overall survival	16 months (CI 13.9–18)	17.2 months (CI 13.5–20.9)	21.3 months (CI 16.9–25.7)
Median progression-free survival	6 months (CI 4.9–7)	7 months (CI 5.8–8.1)	9 months (CI 7.4–10.6)

Gender-wise median OS was 21.5 months in male obese patients (CI 16.5–26.5), 16.2 months (CI 13.7–18.7) in the non-obese ($p=0.217$), 17.6 months (CI 12.4–22.8) in the overweight ($p=0.321$) and 15.5 months in normal-weight males (CI 12.2–18.7) ($p=0.109$).

There was no statistically significant difference in median PFS within male BMI subgroups.

Median OS was 21.3 months (CI 11–31.6) in obese females, 16.3 months (CI 12.7–19.9) in the non-obese ($p=0.076$), 15.7 months (CI 9–22.3) in the overweight ($p=0.160$) and 16.3 (CI 12–20.6) months in normal-weight women (p value = 0.073).

Median PFS was 9.5 months in obese females (CI 5–13.9) and 7 months in normal-weight women (CI 5.2–7.7) (p value = 0.017).

Independent prognostic factors

A multivariate analysis on overall survival with cox-regression including univariate p values < 0.1 was conducted and age < 60 (p value = 0.044; HR 0.73), gross total resection (p value = 0.004; HR 0.62) and body mass index ≥ 30 (p value = 0.009; HR 0.56) were identified as independent prognostic factors associated with longer survival.

Multivariate analysis on progression-free survival did not identify either GTR (p value = 0.066; HR 0.76) or BMI ≥ 30 (p value = 0.083; HR 0.7) as statistically significant independent prognostic factors.

Table 2 Kaplan–Meier pairwise comparison of prognostic factors and cox-regression *p* values for prognostic factors with *p* < 0.1 on univariate analysis

Prognostic factor	Median OS	Median PFS	X ² square OS	X ² square PFS	<i>p</i> value OS	<i>p</i> value PFS	Multivariate analysis <i>p</i> -value
EOR total	19 months (CI 15.8–22.2)	8 months (CI 7.1–8.9)	11.443	4.332	0.001	0.037	0.04 (HR 0.62)/0.066 (HR 0.76)
EOR subtotal	14.6 months (CI 11.1–18)	6 months (CI 5–6.9)					
ECOG 0–1	18 months (CI 15.9–20)	8 months (CI 7–9.1)	2.962	0.702	0.085	0.402	0.67 (HR 0.91)/–
ECOG 2–4	14.6 months (CI 11.5–17.7)	6 months (CI 5–6.9)					
Age < 60 years	19.3 months (CI 17–21.6)	8 months (CI 7–8.9)	5.390	0.842	0.020	0.359	0.044 (HR 0.73)/–
Age > 60 years	14.8 months (CI 13.2–16.4)	6 months (CI 5.2–6.8)					
Obese	21.3 months (CI 16.9–25.7)	9 months (CI 7.4–10.6)	5.687	3.848	0.017	0.050	0.009 (HR 0.56)/0.083 (HR 0.7)
Non-obese	16.2 months (CI 14–18.3)	7 months (CI 6.3–7.7)					
Obese	21.3 months (CI 16.9–25.7)	9 months (CI 7.4–10.6)	7.395	8.166	0.007	0.004	–
Normal weight	16 months (CI 13.9–18)	6 months (CI 4.9–7)					
Obese	21.3 months (CI 16.9–25.7)	9 months (CI 7.4–10.6)	3.625	1.812	0.057	0.178	–
Overweight	17.2 months (CI 13.5–20.9)	7 months (CI 5.8–8.1)					
Overweight	17.2 months (CI 13.5–20.9)	7 months (CI 5.8–8.1)	1.763	3.657	0.184	0.056	–
Normal weight	16 months (CI 13.9–18)	6 months (CI 4.9–7)					
Smoker	14.7 months (CI 6.9–22.5)	7 months (CI 5.7–8.2)	0.049	0.181	0.825	0.670	–
Non-smoker	17.3 months (CI 15.5–19)	7.5 months (CI 6.4–8.6)					
Diabetic	15.2 months (CI 10.7–19.7)	6 months (CI 5.2–6.8)	2.718	1.332	0.099	0.248	0.137 (HR 0.7)/–
Non-diabetic	18 months (CI 15.8–20.2)	8 months (CI 7–8.9)					
Hypertense	15.5 months (CI 12.9–18.1)	8 months (CI 6.4–9.6)	0.802	0.123	0.370	0.726	–
Non-hypertense	18 months (CI 16–19.9)	7 months (CI 5.7–8.3)					

Discussion

Glioblastoma remains a biological and clinical challenge with dismal long-term survival despite best care with maximal extent of resection and chemoradiotherapy. A reduced number of patients are able to live for many years without signs of disease and a few others maintain long-term disease stability after tumour recurrence.

In order to improve patient outcome, it is of paramount importance to identify subgroups of subjects with increased

OS or PFS that share clinical or tumour biological characteristics. Understanding the mechanism behind such phenomenon might lead to better tumour pathogenesis knowledge and help find novel molecular and clinical therapeutic targets.

In a 2018 retrospective series of 392 GBM patients, Potharaju et al. [17] sought to analyse the effect of BMI on overall survival. The overweight and obese patients had statistically significant longer median OS when compared to normal-weight subjects. We report similar results. Statistically significant increased OS and PFS were observed in

Table 3 Statistical comparison between BMI categories regarding overall survival (*p* values in the center squares)

	Normal weight	Overweight	Obese	Non-obese
	Total: 16 months (CI 13.9–18)	Total: 17.2 months (CI 13.5–20.9)	Total: 21.3 months (CI 16.9–25.7)	Total: 16.2 months (CI 14–18.3)
	Males: 15.5 months (CI 12.2–18.7)	Males: 17.6 months (CI 12.4–22.8)	Males: 21.5 months (CI 16.5–26.5)	Males: 16.2 months (CI 13.7–18.7)
	Females: 16.3 months (CI 12–20.6)	Females: 15.7 months (CI 9–22.3)	Females: 21.3 months (CI 11–31.6)	Females: 16.3 months (CI 12.7–19.9)
Normal weight				
Total: 16 months (CI 13.9–18)	–	0.184	0.007	–
Males: 15.5 months (CI 12.2–18.7)		0.201	0.109	
Females: 16.3 months (CI 12–20.6)		0.574	0.073	
Overweight				
Total: 17.2 months (CI 13.5–20.9)	0.184	–	0.057	–
Males: 17.6 months (CI 12.4–22.8)	0.201		0.321	
Females: 15.7 months (CI 9–22.3)	0.574		0.160	
Obese				
Total: 21.3 months (CI 16.9–25.7)	0.007	0.057	–	0.017
Males: 21.5 months (CI 16.5–26.5)	0.109	0.321		0.217
Females: 21.3 months (CI 11–31.6)	0.073	0.160		0.076
Non-obese				
Total: 16.2 months (CI 14–18.3)	–	–	0.017	–
Males: 16.2 months (CI 13.7–18.7)			0.217	
Females: 16.3 months (CI 12.7–19.9)			0.076	

Table 4 Statistical comparison between BMI categories regarding progression-free survival (*p* values in the center squares)

	Normal weight	Overweight	Obese	Non-obese
	Total: 6 months (CI 4.9–7)	Total: 7 months (CI 5.8–8.1)	Total: 9 months (CI 7.4–10.6)	Total: 7 months (CI 6.3–7.7)
	Males: 6 months (CI 5–7)	Males: 7.5 months (CI 6.3–8.6)	Males: 9 months (CI 7.4–10.6)	Males: 7 months (CI 5.6–8.4)
	Females: 7 months (CI 5.2–7.7)	Females: 6 months (CI 5.7–6.3)	Females: 9.5 months (CI 5–13.9)	Females: 6 months (CI 5–6.9)
Normal weight				
Total: 6 months (CI 4.9–7)	–	0.056	0.004	–
Males: 6 months (CI 5–7)		0.172	0.272	
Females: 7 months (CI 5.2–7.7)		0.231	0.017	
Overweight				
Total: 7 months (CI 5.8–8.1)	0.056	–	0.178	–
Males: 7.5 months (CI 6.3–8.6)	0.172		0.696	
Females: 6 months (CI 5.7–6.3)	0.231		0.399	
Obese				
Total: 9 months (CI 7.4–10.6)	0.004	0.178	–	0.050
Males: 9 months (CI 7.4–10.6)	0.272	0.696		0.524
Females: 9.5 months (CI 5–13.9)	0.017	0.399		0.119
Non-obese				
Total: 7 months (CI 6.3–7.7)	–	–	0.050	–
Males: 7 months (CI 5.6–8.4)			0.524	
Females: 6 months (CI 5–6.9)			0.119	

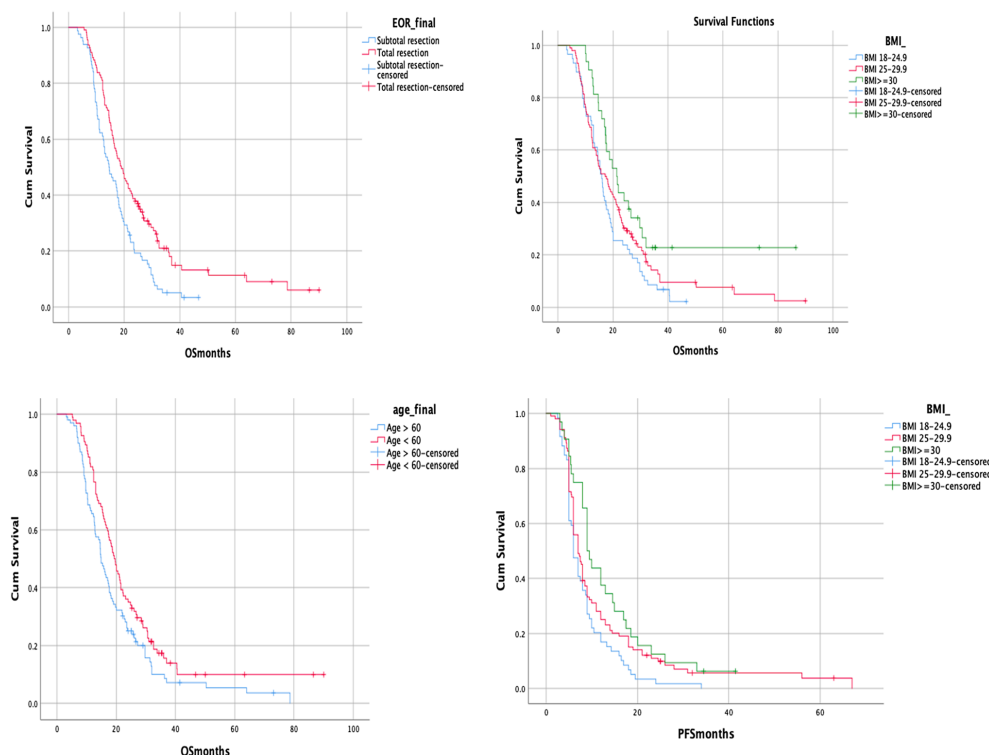


Fig. 1 Overall survival functions for EOR, BMI category and age. On the bottom right, survival curve for PFS according to BMI subset

obese patients in comparison to normal-weight subjects, and increasing BMI was associated with higher OS.

When adjusted for other prognostic factors on multivariate analysis, a BMI ≥ 30 kg/m² had a statistically significant positive impact on outcome, similar to gross total resection and younger age.

BMI and cancer link

The term “obesity paradox” has been used to describe the longer cancer survival of overweight and obese patients even though excess body weight is associated with increased cancer risk.

Some authors [18–20] have tried to explain those findings by pinpointing to methodological flaws of previous studies, namely inadequacy of BMI to determine body composition, existence of confounders, detection bias and reverse causation. Park et al. [18] and Lennon and colleagues [19] argue that body mass index is not the ideal measurement of body fat and better predictors of adiposity and body composition should be used before establishing a link between BMI and increased survival. Whilst true,

body fat measurement with BMI still remains the clinical benchmark as other methods are at the current time not suitable for clinical practice.

In our study, the obese subgroup had better OS and PFS outcomes, and while body composition might be harder to gauge in the overweight, excess adiposity is almost certain in the obese.

Furthermore, BMI was the clinical marker used to link excess body weight and adiposity to cancer risk in multiple studies [21–23].

Confounders such as smoking status and comorbidities have also been used to try to explain the obesity paradox.

In our study, there was no statistically significant difference between groups in smoking and ECOG performance status and as expected cardiovascular risk factors were more frequent in the overweight and obese subgroups; thus, our overweight and obese patients were unlikely to be healthier than normal-weight individuals. Median age for the obese and overweight subgroups was also higher.

Unsurprisingly, detection bias does not apply to glioblastoma as the natural history of the disease is rapid and as such reverse causation is also less likely to be a factor in

glioblastoma patients as GBM-induced weight loss is less exuberant than other cancers.

At our institution and with few exceptions, glioblastomas are operated within a week of MRI-imaging diagnosis and steroids are usually tapered off post-operatively during a 5–10 day period. Therefore, steroid usage is unlikely to have significantly impacted BMI.

Socioeconomical disparity between groups is also unlikely to explain the difference in OS and PFS as all patients received treatment in a public hospital, where healthcare access is free and universal.

We believe there may be other factors besides heterogeneity within different BMI groups and the lack of a more effective means to establish body composition in standard clinical practice that can justify the longer survival of overweight and obese cancer patients observed in various studies.

Higher BMI and cancer survival

Multiple studies have reported increased survival of overweight and obese subjects relative to under and normal-weight individuals after cancer diagnosis [24, 25].

In their evaluation of BMI and outcome in colorectal cancer, Shahjehan et al. [26] demonstrated that being underweight was a negative predictor of outcome and when all stages of cancer were combined obese and overweight subjects lived longer.

McQuade et al. [27] found an association between increased survival and obesity in male patients under targeted and immune therapies for metastatic melanoma while Choi et al. [28], in a meta-analysis of nephrectomised renal cell cancer (RCC) patients, identified preoperative obesity as an independent prognostic factor for increased OS and cancer-specific survival (CSS).

Rodge et al. [29] found that notwithstanding more frequent post-op complications, obese patients had a statistically significant increased CSS among RCC patients subjected to surgery, and Schrader and colleagues [30] found an association between being overweight and improved CSS in organ confined RCC.

In patients with good performance status, Tsang et al. [31] demonstrated obesity to be linked to less mortality in patients with distant metastases from varied neoplasms.

Recent studies have tried to understand the reason behind higher BMI and better cancer outcomes.

In a cohort study from five different clinical studies, Sanchez et al. [32] aimed to assess differences in transcriptomics between tumour and peritumour tissue of both local and metastasized clear cell RCC of normal-weight and obese patients. Differences in tumour microenvironment were found with higher angiogenic scores and increased peritumoural inflammation encountered in specimen from obese individuals.

Albiges et al. [33] reported increased OS in obese RCC patients and established an inverse relationship between fatty acid synthase (FASN) expression and increased survival; FASN gene expression was downregulated in obese patients.

FASN has been linked to altered tumour biological behaviour both in RCC and gliomas [34].

Hakimi et al. [35] showed higher BMI to be linked to increased OS in RCC on univariate analysis, and most importantly, genome-wide studies revealed statistically significant upregulation of FASN in normal-weight patients while downregulated in obese individuals. In that study, FASN overexpression was also associated with decreased survival.

Zhao et al. [36] found fatty acid synthase to be increased in glioma cells while Grube and colleagues [37] reported a linear relationship between increased FASN expression and higher tumour grade and propose FASN inhibition as a target for glioma therapy.

Given the aforementioned findings regarding tumour biology of different neoplasms, we hypothesize that tumour microenvironment might be altered in obese patients, in particular with regard to fatty acid metabolism and angiogenesis.

Thus, the longer survival and progression-free survival experienced by our obese glioblastoma patient subgroup might be explained by obesogenic-induced tumour microenvironment change.

Better tolerance to chemoradiotherapy, higher energy reserves and lower tendency for cachexia [38] might have also contributed to the trend of increasing BMI associated with higher survival observed in our study.

General considerations

Our study is not devoid of shortcomings: it is a retrospective study, MGMT promoter gene methylation status was not accounted for and albeit the most used in clinical practice, BMI still remains an imperfect measurement of body adiposity, particularly in the overweight that constitutes the main bulk of our patients.

Conclusion

Excess body weight and obesity remain a public health problem due to the negative impact on cardiovascular health and increased cancer risk.

However, in similar fashion to multiple studies in various neoplasms, our glioblastoma patients with higher BMI fared better in terms of overall survival and progression-free survival than normal-weight patients.

Consequently, establishing a definitive link between excess body weight and increased cancer survival in prospective studies is of crucial importance.

Transcriptomics, proteomics and metabolomics are among the tools currently available to ascertain differences in glioblastoma microenvironment of obese patients that might explain the more indolent disease course. A better understanding of tumour pathogenesis and biological behaviour within this subgroup might result in better patient outcome through identification of novel molecular and therapeutic targets.

Author contributions Guarantor of integrity of the entire study: PVA. Study concepts and design: PVA and PL. Literature research: PVA and PL. Clinical studies: PVA. Experimental studies/data analysis: PVA and BDC. Statistical analysis: PVA and BDC. Manuscript preparation: PVA, PL and RV. Manuscript editing: PL and RV.

Data availability All datasets generated for this manuscript can be obtained from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. Tamimi AF, Juweid M (2017) Epidemiology and outcome of glioblastoma. *Glioblastoma*. Codon Publications, Brisbane, pp 143–153
2. Sanai N, Polley MY, McDermott MW, Parsa AT, Berger MS (2011) An extent of resection threshold for newly diagnosed glioblastomas. *J Neurosurg* 115(1):3–8. <https://doi.org/10.3171/2011.2.jns.10998>
3. World Health Organization (2018) Obesity and overweight fact sheet. World Health Organization, Geneva
4. Sung H, Siegel RL, Torre LA, Pearson-Stuttard J, Islami F, Fedewa SA, Goding Sauer A, Shuval K, Gapstur SM, Jacobs EJ, Giovannucci EL, Jemal A (2019) Global patterns in excess body weight and the associated cancer burden. *CA* 69:88–112. <https://doi.org/10.3322/caac.21499>
5. Muskens IS, Wu AH, Porcel J, Cheng I, Le Marchand L, Wiemels JL, Setiawan VW (2019) Body mass index, comorbidities, and hormonal factors in relation to meningioma in an ethnically diverse population: the Multiethnic Cohort. *Neuro-Oncology* 21(4):498–507. <https://doi.org/10.1093/neuonc/now005>
6. Wiedmann M, Brunborg C, Lindemann K, Johannesen TB, Vatten L, Helseth E, Zwart J (2013) Body mass index and the risk of meningioma, glioma and schwannoma in a large prospective cohort study (The HUNT Study). *Br J Cancer* 109(1):289–294. <https://doi.org/10.1038/bjc.2013.304>
7. Kolb R, Sutterwala FS, Zhang W (2016) Obesity and cancer: inflammation bridges the two. *Curr Opin Pharmacol* 29:77–89. <https://doi.org/10.1016/j.coph.2016.07.005>
8. Cirillo F, Catellani C, Sartori C, Lazzeroni P, Amarri S, Street ME (2019) Obesity, insulin resistance, and colorectal cancer: could miRNA dysregulation play a role? *Int J Mol Sci* 20(12):2922. <https://doi.org/10.3390/ijms20122922>
9. Parekh N, Chandran U, Bandera EV (2012) Obesity in cancer survival. *Annu Rev Nutr* 32:311–342. <https://doi.org/10.1146/annurev-nutr-071811-150713>
10. Demark-Wahnefried W, Platz EA, Ligibel JA, Blair CK, Courneya KS, Meyerhardt JA, Ganz PA, Rock CL, Schmitz KH, Wadden T, Philip EJ, Wolfe B, Gapstur SM, Ballard-Barbash R, McTiernan A, Minasian L, Nebeling L, Goodwin PJ (2012) The role of obesity in cancer survival and recurrence. *Cancer Epidemiol Biomark Prev* 21(8):1244–1259. <https://doi.org/10.1158/1055-9965.epi-12-0485>
11. Niedermaier T, Behrens G, Schmid D, Schlecht I, Fischer B, Leitzmann MF (2015) Body mass index, physical activity, and risk of adult meningioma and glioma: a meta-analysis. *Neurology* 85(15):1342–1350. <https://doi.org/10.1212/WNL.00000000000002020>
12. Saunders CN, Cornish AJ, Kinnarsley B, Law PJ, Claus EB, Il'yasova D, Schildkraut J, Barnholtz-Sloan JS, Olson SH, Bernstein JL, Lai RK, Chanock S, Rajaraman P, Johansen C, Jenkins RB, Melin BS, Wrensch MR, Sanson M, Bondy ML, Houlston RS (2020) Lack of association between modifiable exposures and glioma risk: a Mendelian randomization analysis. *Neuro Oncol* 22(2):207–215. <https://doi.org/10.1093/neuonc/now209>
13. Wiedmann M, Brunborg C, Di Ieva A, Lindemann K, Johannesen TB, Vatten L, Helseth E, Zwart JA (2017) The impact of body mass index and height on the risk for glioblastoma and other glioma subgroups: a large prospective cohort study. *Neuro-Oncology* 19(7):976–985. <https://doi.org/10.1093/neuonc/now272>
14. Siegel EM, Nabors LB, Thompson RC, Olson JJ, Browning JE, Madden MH, Han G, Egan KM (2013) Prediagnostic body weight and survival in high grade glioma. *J Neurooncol* 114(1):79–84. <https://doi.org/10.1007/s11060-013-1150-2>
15. Jones LW, Ali-Osman F, Lipp E, Marcello JE, McCarthy B, McCoy L, Rice T, Wrensch M, Il'yasova D (2010) Association between body mass index and mortality in patients with glioblastoma multiforme. *Cancer Causes Contr* 21(12):2195–2201. <https://doi.org/10.1007/s10552-010-9639-x>
16. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352:987–996. <https://doi.org/10.1056/NEJMoa043330>
17. Potharaju M, Mangaleswaran B, Mathavan A, John R, Thamburaj V, Ghosh S, Ganesh S, Kalvakonda C, Loganathan M, Bapu S, Devi R, Verma RS (2018) Body mass index as a prognostic marker in glioblastoma multiforme: a clinical outcome. *Int J Radiat Oncol Biol Phys* 102(1):204–209. <https://doi.org/10.1016/j.ijrobp.2018.05.024>
18. Park Y, Peterson LL, Colditz GA (2018) The plausibility of obesity paradox in cancer-point. *Can Res* 78(8):1898–1903. <https://doi.org/10.1158/0008-5472.CAN-17-3043>
19. Lennon H, Sperrin M, Badrick E, Renehan AG (2016) The obesity paradox in cancer: a review. *Curr Oncol Rep* 18(9):56. <https://doi.org/10.1007/s11912-016-0539-4>
20. Lee DH, Giovannucci EL (2019) The obesity paradox in cancer: epidemiologic insights and perspectives. *Curr Nutr Rep* 8(3):175–181. <https://doi.org/10.1007/s13668-019-00280-6>
21. Secord AA, Hasselblad V, Von Gruenigen VE, Gehrig PA, Modesitt SC, Bae-Jump V, Havrilesky LJ (2016) Body mass

- index and mortality in endometrial cancer: a systematic review and meta-analysis. *Gynecol Oncol* 140(1):184–190. <https://doi.org/10.1016/j.ygyno.2015.10.020>
22. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M (2008) Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* 371(9612):569–578. [https://doi.org/10.1016/S0140-6736\(08\)60269-X](https://doi.org/10.1016/S0140-6736(08)60269-X)
 23. Wang J, Yang DL, Chen ZZ, Gou BF (2016) Associations of body mass index with cancer incidence among populations, genders, and menopausal status: a systematic review and meta-analysis. *Cancer Epidemiol* 42:1–8. <https://doi.org/10.1016/j.canep.2016.02.010>
 24. Arkenbosch JHC, van Erning FN, Rutten HJ, Zimmerman D, de Wilt JHW, Beijer S (2018) The association between Body Mass Index and postoperative complications, 30-day mortality and long-term survival in Dutch patients with colorectal cancer. *Eur J Surg Oncol* 45(2):160–166. <https://doi.org/10.1016/j.ejso.2018.09.012>
 25. Jeon HG, Jeong IG, Lee JH, Lee CJ, Kwak C, Kim HH, Lee ES, Lee E (2010) Prognostic value of body mass index in Korean patients with renal cell carcinoma. *J Urol* 183(2):448–454. <https://doi.org/10.1016/j.juro.2009.10.004>
 26. Shahjehan F, Merchea A, Cochuyt JJ, Li Z, Colibaseanu DT, Kasi PM (2018) Body mass index and long-term outcomes in patients with colorectal cancer. *Front Oncol* 8:620. <https://doi.org/10.3389/fonc.2018.00620>
 27. McQuade JL, Daniel CR, Hess KR, Mak C, Wang DY, Rai RR, Park JJ, Haydu LE, Spencer C, Wongchenko M, Lane S, Lee DY, Kaper M, McKean M, Beckermann KE, Rubinstein SM, Rooney I, Musib L, Budha N, Hsu J, Nowicki TS, Avila A, Haas T, Puligandla M, Lee S, Fang S, Wargo JA, Gershenwald JE, Lee JE, Hwu P, Chapman PB, Sosman JA, Schadendorf D, Grob JJ, Flaherty KT, Walker D, Yan Y, McKenna E, Legos JJ, Carlino MS, Ribas A, Kirkwood JM, Long GV, Johnson DB, Menzies AM, Davies MA (2018) Association of body-mass index and outcomes in patients with metastatic melanoma treated with targeted therapy, immunotherapy, or chemotherapy: a retrospective, multicohort analysis. *Lancet Oncol* 19(3):310–322. [https://doi.org/10.1016/S1470-2045\(18\)30078-0](https://doi.org/10.1016/S1470-2045(18)30078-0)
 28. Choi Y, Park B, Jeong BC, Seo SI, Jeon SS, Choi HY, Lee HM (2012) Body mass index and survival in patients with renal cell carcinoma: a clinical-based cohort and meta-analysis. *Int J Cancer* 132(3):625–634. <https://doi.org/10.1002/ijc.27639>
 29. Rogde AJ, Gudbrandsdottir G, Hjelle KM, Sand KE, Bostad L, Beisland C (2012) Obesity is associated with an improved cancer-specific survival, but an increased rate of postoperative complications after surgery for renal cell carcinoma. *Scand J Urol Nephrol* 46(5):348–357. <https://doi.org/10.3109/00365599.2012.678382>
 30. Schrader AJ, Rustemeier J, Rustemeier JC, Timmesfeld N, Varga Z, Hegele A, Olbert PJ, Hofmann R (2009) Overweight is associated with improved cancer-specific survival in patients with organ-confined renal cell carcinoma. *J Cancer Res Clin Oncol* 135:1693–1699. <https://doi.org/10.1007/s00432-009-0616-2>
 31. Tsang NM, Pai PC, Chuang CC, Chuang WC, Tseng CK, Chang KP, Yen TC, Lin JD, Chang JT (2016) Overweight and obesity predict better overall survival rates in cancer patients with distant metastases. *Cancer Med* 5(4):665–675. <https://doi.org/10.1002/cam4.634>
 32. Sanchez A, Furberg H, Kuo F, Vuong L, Ged Y, Patil S, Ostrovaya I, Petruzella S, Reising A, Patel P, Mano R, Coleman J, Russo P, Liu HC, Dannenberg JA, Chan AT, Motzer R, Voss H, Hakimi AA (2019) Transcriptomic signatures related to the obesity paradox in patients with clear cell renal cell carcinoma: a cohort study. *Lancet Oncol* 21(2):283–293. [https://doi.org/10.1016/S1470-2045\(19\)30797-1](https://doi.org/10.1016/S1470-2045(19)30797-1)
 33. Albiges L, Hakimi AA, Xie W, McKay RR, Simantov R, Lin X, Lee JL, Rini BI, Srinivas S, Bjarnason GA, Ernst S, Wood LA, Vaishamayan UN, Rha SY, Agarwal N, Yuasa T, Pal SK, Bamias A, Zabor EC, Skanderup AJ, Furberg H, Fay AP, de Velasco G, Preston MA, Wilson KM, Cho E, McDermott DF, Signoretti S, Heng DY, Choueiri TK (2016) Body mass index and metastatic renal cell carcinoma: clinical and biological correlations. *J Clin Oncol* 34(30):3655–3663. <https://doi.org/10.1200/JCO.2016.66.7311>
 34. Horiguchi A, Asano T, Asano T, Ito K, Sumitomo M, Hayakawa M (2008) Fatty acid synthase over expression is an indicator of tumor aggressiveness and poor prognosis in renal cell carcinoma. *J Urol* 180(3):1137–1140. <https://doi.org/10.1016/j.juro.2008.04.135>
 35. Hakimi AA, Furberg H, Zabor EC, Jacobsen A, Schultz N, Ciriello G, Mikkilineni N, Fiegoli B, Kim PH, Voss MH, Shen H, Laird PW, Sander C, Reuter VE, Motzer RJ, Hsieh JJ, Russo P (2013) An epidemiologic and genomic investigation into the obesity paradox in renal cell carcinoma. *J Natl Cancer Inst* 105(24):1862–1870. <https://doi.org/10.1093/jnci/djt310>
 36. Zhao W, Kridel S, Thorburn A, Kooshki M, Little J, Hebbard S, Robbins M (2006) Fatty acid synthase: a novel target for anti-glioma therapy. *Br J Cancer* 95:869–878. <https://doi.org/10.1038/sj.bjc.6603350>
 37. Grube S, Dünisch P, Freitag D, Klausnitzer M, Sakr Y, Walter J, Kalff R, Ewald C (2014) Overexpression of fatty acid synthase in human gliomas correlates with the WHO tumor grade and inhibition with Orlistat reduces cell viability and triggers apoptosis. *J Neurooncol* 118:277–287. <https://doi.org/10.1007/s11060-014-1452-z>
 38. Zhang X, Liu Y, Shao H, Zheng X (2017) Obesity paradox in lung cancer prognosis: evolving biological insights and clinical implications. *J Thorac Oncol* 12(10):1478–1488. <https://doi.org/10.1016/j.jtho.2017.07.022>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.