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Crosstalk between the immune system and the EMT

CADHERINS AS BIOMARKERS IN GLIOBLASTOMA

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TESE DE DOUTORAMENTO APRESENTADA
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CROSSTALK BETWEEN THE IMMUNE SYSTEM AND THE EMT – cadherins as biomarkers in Glioblastoma

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Instituições de acolhimento

Artigo 48º, § 3º - A Faculdade não responde pelas doutrinas expendidas na Dissertação.

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List of Publications

The worked developed within the scope of this thesis led to the following publications:

- **Noronha C**, Ribeiro AS, Taipa R, Leitão D, Schmitt F, Reis J, Faria C, Paredes J. PD-L1 tumor expression is associated with poor prognosis and systemic immunosuppression in glioblastoma. J Neurooncol 156(3): 453-464, 2022. doi: 10.1007/s11060-021-03907-3.

- **Noronha C**, Ribeiro AS, Carvalho R, Mendes N, Reis J, Faria C, Taipa R, Paredes J. Cadherin expression profiles define glioblastoma differentiation and patient prognosis. Cancers, 16(13): 2298, 2024. doi: 10.3390/cancers16132298

- **Noronha C,** Pinto C, Fernandes A, Magalhães M, Ramos C, Pinheiro C, Silva E, Calheiros A, Taipa R, Reis J. Exploring Clinical Prognosticators in a series of 313 Glioblastoma patients: Insights and Implications, 2025. *(Submitted for publication)*

- **Noronha C,** Ribeiro AS, Mendes N, Reis J, Faria C, Castro D, Taipa R, Paredes J. Cadherins as biomarkers of ZEB/PD-L1 interactions in Glioblastoma. 2025 *(Submitted for publication)*

The following review article, although not an integral part of this dissertation, was used in the introduction of the thesis:

- **Noronha C**, Ribeiro AS, Taipa R, Castro DS, Reis J, Faria C, Paredes J. Cadherin Expression and EMT: A Focus on Gliomas. Biomedicines 9(10): 1328, 2021. doi: 10.3390/biomedicines9101328.

Abbreviations

ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
APC	antigen-presenting cells
ATRX	alpha-thalassemia/mental retardation, X-linked
BBB	blood brain barrier
BCNU	bis-chloroethylnitrosourea
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BTLA	B- and T-Lymphocyte attenuator
CAR	chimeric antigen receptor
CBTRUS	Central Brain Tumor Registry of the United States
CC	corpus callosum
CCDC26	coiled-coil domain containing 26
CCNE1	cyclin E1
CDH1	E-cadherin gene
CDH2	N-cadherin gene
CDH3	P-cadherin gene
CDKN2B	cyclin dependent kinase inhibitor 2B
CET	contrast-enhancing tumor
CNS	central nervous system
CSC	cancer stem cells
CSF	cerebral spinal fluid
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DCE	dynamic contrast enhanced
DNA	deoxyribonucleic acid
DSC	dynamic susceptibility contrast
DTI	diffusion tensor imaging
DWI	diffusion weighted imaging
E-cad	E-cadherin
EGFR	epidermal growth factor receptor
EMP	epithelial-mesenchymal plasticity
EMT	epithelial to mesenchymal transition
EOR	extent of resection
EORTC	European Organization for Research and Treatment of Cancer
Ep-GBM	epithelioid glioblastoma
ERK	extracellular signal-regulated kinase
FFPE	formalin-fixed and paraffin-embedded
FGFR	fibroblast growth factor receptor
GBM	glioblastoma
GS	gliosarcoma
GTR	gross-total resection

GWAS	genome-wide association studies
ICP	immune checkpoints
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
IL-10	interleukin-10
JAK	janus kinase
KPS	Karnofsky Performance Status
LAG3	lymphocyte activation gene 3
LITT	laser interstitial thermal therapy
MAPK	mitogen-activated protein kinase
MET	mesenchymal to epithelial transition
MDSC	myeloid-derived suppressor cells
MGMT	methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MRI	Magnetic Resonance Imaging
mRNA	messenger ribonucleic acid
MRS	Magnetic Resonance Spectroscopy
N-cad	N-cadherin
NF1	neurofibromin 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	neutrophil-to-lymphocyte ratio
OS	overall survival
P-cad	P-cadherin
PD-1	programmed cell death protein-1
PD-L1	programmed cell death ligand-1
PDGFRA	platelet-derived growth factor receptor A
PFS	progression-free survival
PHLDB1	pleckstrin homology-like domain family B member 1
PI3K	phosphoinositide 3-kinase
PR	partial resection
PTEN	phosphatase and tensin homolog
QTRT	chemotherapy plus radiotherapy
RB1	retinoblastoma protein
ROBO1	roundabout homolog 1
RT	radiotherapy
RTEL1	regulator of telomere elongation helicase 1
RT-PCR	real-time polymerase chain reaction
SLAMF6	signaling lymphocytic activation molecule family member 6
SNAIL	zinc finger protein SNAI1
SNP	single nucleotide polymorphism
SOX2	sex determining region Y-box 2
STAG2	cohesin subunit SA-2

STAT	signal transducer and activator of transcription
STR	subtotal resection
SVZ	subventricular zone
TAM	tumor-associated macrophages
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TF	transcription factor
TGFß	transforming growth factor-ß
TIGIT	T-cell Immunoreceptor with Ig and ITIM domains
TIL	tumor infiltrating lymphocyte
TIM3	T-cell Immunoglobulin and Mucin Domain-Containing Protein 3
TK	tyrosine kinase
TMA	tissue microarrays
TMZ	temozolomide
TP53	tumor protein 53
Tregs	regulatory T cells
TWIST	Twist-related protein 1
VASARI	Visually Accessible REMBRANDT Images
WHO	World Health Organization
ZEB	zinc finger E-box-binding homeobox
2B4	Natural Killer Cell Receptor 2B4

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Summary

Glioblastoma (GBM) is the most common primary malignant brain tumor and one of the most lethal and challenging cancers. While innovative therapies have significantly improved clinical outcomes in other cancers, clinical trials for GBM have thus far yielded only modest results. This limited progress is due to GBM unique characteristics, including its intracranial location, rapid progression, local invasiveness, and high intratumoral heterogeneity. Another major challenge remains the identification of relevant biomarkers for GBM.

Emerging data suggests that two prominent cancer mechanisms may interact to drive GBM aggressiveness: the activation of the epithelial-to-mesenchymal transition (EMT) program and concomitant induction of immunosuppression. Importantly, while GBM is notorious for its profound immunosuppression, the role and functional significance of an EMT-like program in gliomagenesis is less understood. It is therefore important to investigate the presence of an EMT program in GBM and its interplay with the GBM-induced immunosuppression. In addressing these questions, we also aimed to identify putative disease-relevant biomarkers.

Using a retrospective cohort of 313 GBM patients, it is first demonstrated that local and systemic immunosuppression portends a poor prognosis for GBM patients. Notably, PD-L1 expression, a key mechanism of immune evasion, was upregulated in IDH wild-type tumors with sarcomatous dedifferentiation. Analysis of classical cadherins expression in the same cohort, recognized as EMT markers in epithelial cancers, allowed us to identify P-cadherin as a prominent marker for worse prognosis and treatment resistance in GBM. Moreover, we propose that the combined expression of E-, N-, and P-cadherin improved the identification of biologically relevant GBM subgroups with distinct clinical, imaging, neuropathological, and survival characteristics. We further investigated ZEB1 and ZEB2 transcription factors expression and documented high expression rates for these transcription factors, with an important increase upon tumor recurrence. Finally, integrating the expression data of ZEB1/ZEB2 with cadherins profiles allowed the refinement of the proposed GBM subgroups, which

appear to represent distinct EMT states. These subgroups were also associated to differences in PD-L1 expression and systemic immunosuppression/inflammation.

Altogether, these findings highlight the significant role of EMT and immune evasion mechanisms, particularly involving the PD-1/PD-L1 axis, in GBM pathogenesis and aggressiveness. In addition, they underscore the potential of cadherins expression as valuable biomarkers in GBM. Future studies should focus on elucidating the molecular mechanisms underlying the interplay between ZEB1/ZEB2 and cadherins, with the goal of translating these insights into potential therapeutic targets and improving prognosis prediction in GBM.

Resumo

O glioblastoma (GBM) é o tumor cerebral maligno primário mais comum no adulto e um dos cancros mais letais, contribuindo com uma taxa de mortalidade desproporcional à sua taxa de incidência. Os desafios apresentados por este tumor reflectem a sua localização no Sistema Nervoso Central, bem como a sua elevada heterogeneidade, capacidade de invasão e resistência ao tratamento. Nas últimas décadas, a introdução de várias terapêuticas inovadoras levou a uma significativa melhoria nos resultados clínicos em vários cancros sistémicos. Porém, no GBM, os ensaios clínicos até agora realizados têm conseguido apenas resultados modestos. Um importante desafio neste tumor é a identificação de biomarcadores que sejam relevantes para a estratificação do prognóstico e decisão terapêutica.

A ativação do programa de transição epitelial-mesenquimal (EMT) e a indução de imunossupressão são dois mecanismos chave no desenvolvimento de vários cancros sistémicos e que têm sido recentemente investigados no GBM. Vários estudos propõem que estes mecanismos poderão interagir e contribuir conjuntamente para a agressividade deste tumor. Se por um lado a imunossupressão é já aceite como um mecanismo inequivocamente envolvido na patogénese do GBM, o papel e a função de um programa semelhante ao EMT num tumor não epitelial ainda necessitam de adequada investigação e validação.

O nosso principal objetivo foi investigar a presença de um programa de EMT no GBM, bem como documentar a interação com a imunossupressão por ele induzida. Concomitantemente, procurámos identificar biomarcadores da doença.

Usando uma coorte retrospetiva de 313 doentes com GBM, demonstrámos que a imunossupressão local e sistémica se associa significativamente com um prognóstico desfavorável. Em particular, a expressão de PD-L1, um mecanismo de evasão ao sistema imune, está aumentada em tumores IDH *wildtype* e naqueles com desdiferenciação sarcomatosa. A avaliação da expressão das caderinas clássicas, conhecidas como marcadores de EMT em carcinomas, permitiu-nos identificar a P-caderina como um

proeminente marcador de mau prognóstico e resistência ao tratamento complementar no GBM. A expressão combinada de E-, N- e P-caderina permitiu a identificação de subgrupos biologicamente relevantes distinguidos por diferentes características clínicas, imagiológicas, neuropatológicas e de sobrevivência. A expressão dos fatores de transcrição ZEB1/ZEB2 também foi avaliada, revelando elevadas taxas de expressão e um aumento significativo nas recidivas tumorais. Por último, a integração da expressão de ZEB1/ZEB2 com a expressão de caderinas permitiu-nos melhor caracterizar e compreender estes diferentes subgrupos. Estes parecem reflectir não só diferentes estados de EMT, mas também diferentes graus de imunossupressão/inflamação.

No seu conjunto, estes resultados apontam para um significativo contributo dos mecanismos de EMT e evasão ao sistema imune, em particular envolvendo o eixo PD-1/PD-L1, na gliomagénese e agressividade do GBM. Apoiam ainda a expressão das caderinas como potenciais biomarcadores no GBM. Estudos futuros permitirão elucidar os mecanismos moleculares que ligam a expressão de ZEB1/ZEB2, caderinas e PD-L1, com o objetivo de traduzir estes resultados em potenciais alvos terapêuticos e melhorar a avaliação do prognóstico no GBM.

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CHAPTER I. Introduction

1. Glioblastoma

1.1. Epidemiology, demographics and risk factors

Glioblastoma (GBM) is the most common malignant primary brain tumor in the adult, accounting for 14.2% of all central nervous system (CNS) tumors and 50.1% of the malignant ones [1,2]. In the global panorama of cancers, GBM accounts for 1.4% of world-wide cancer cases with a disproportionate rate of cancer-related deaths of 2.9% [3]. The annual incidence of GBM has been estimated to be 3.26 per 100.000 in the United States [1], with similar rates reported in Europe and lower rates in Japan [4,5]. The risk for this tumor appears to increase steadily with age, reaching a peak of incidence of 15.17 per 100.000 at the age of 75 to 84 years [1].

For the majority of GBM patients, there is no established cause for the disease. A small minority of patients (<5%) has been identified as familiar cases of GBM, with a Mendelian inherited syndrome (such as Lynch syndrome, neurofibromatosis or tuberous sclerosis) identified in approximately 1-2% of adult gliomas [6–10]. Adult glioma incidence is most likely determined by a polygenic model, an explanation proposed by segregation analyses and supported by results from genome-wide association studies (GWAS) [7,11–13]. Since the first reports in 2009, these studies have identified germ line variants associated with glioma susceptibility. Glioma-risk SNPs in have been described in various genes, including *TERT*, regulator of telomere elongation helicase 1 (*RTEL1*), *EGFR*, *TP53*, *CDKN2A/B*, pleckstrin homology-like domain family B member 1 (*PHLDB1*) and coiled-coil domain containing 26 (*CCDC26*) [14–19].

The causality between various environmental risk factors and GBM has been explored including cell phone exposure [20] and viral triggers [21–23]; however, only exposure to ionizing radiation in medium-to-high doses was consistently identified as an exposure risk for GBM [24]. In contrast, case-control studies show that a history of atopic diseases may

decrease the risk for gliomas/GBM [25,26].

GBM has a reported median age of 64 years old [27]; however, with the increase in human longevity in developed countries, a rise in diagnosis is expected in the elderly population. GBM diagnosis is more common in males (1.6 times) and in caucasians (1.98 times)[2,28].

1.2. Clinical presentation

Diagnosis of GBM typically follows the onset of focal neurological deficits, seizures or refractory headache. Neurological symptoms often reflect the involved functional areas of the brain, including motor or speech deficits [29,30]. Other times, presentation includes subtle symptoms, such as executive dysfunction and personality changes, which frequently delay clinical evaluation until a decline in patient autonomy is noticeable [31]. Frequently, mild-to-moderate headaches accompany these symptoms or are the sole presentation in up to half of the patients [32]. They may also be severe and associated with a significant mass effect from the tumor or peri-tumoral edema. Seizures occur in approximately 25% of GBM patients, are less frequently observed than in low-grade glioma patients and usually easier to control[33–36].

GBM is considered to have an initial exponential growth, followed by a plateau phase, which is most probably predicted by a Gompertzian [37] or a stochastic growth model [38].

1.3. Imaging characteristics

The gold standard for the imaging diagnosis of GBM is the structural magnetic resonance imaging (MRI). It typically shows an intra-axial, infiltrative and heterogeneous, ring-enhancing lesion with central necrosis and frequently surrounded by peri-tumoral edema. The presence of cysts, hemorrhage, various foci of contrast-enhancement and, more infrequently,

calcifications, contribute to GBM heterogeneity [39]. These lesions are typically unifocal lesions, with frontal and temporal lobe involvement. These and other tumor qualitative imaging characteristics have been evaluated to predict patient prognosis [40–44] **(Figure 1)**. VASARI (Visually Accessible Rembrandt Images) is a comprehensive set of qualitative and semi-quantitative features for the evaluation of GBM, which contains standardized terminologies of the most common features used to describe primary brain neoplasia on standard pre- and post-contrast enhanced MRI [41,45].

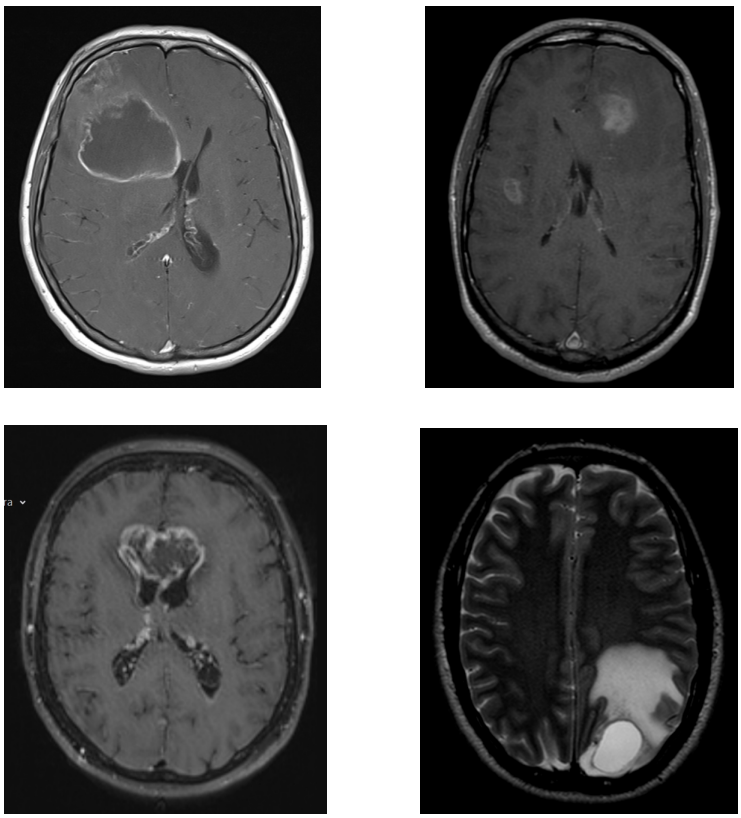

Figure 1. Examples of Glioblastoma MRI features. Examples of MRI features of GBM including the characteristic heterogeneous lesion with peripheral contrast enhancement and central necrosis; multifocal GBM; corpus callosum extension with bilateral contrast-enhancement; and the presence of cysts. Data retrieved from ULS Santo Antonio (DEFI 104-17 (093-DEFI/091-CES).

More recently, advanced MRI techniques (Diffusion Weighted Imaging (DWI), Diffusion Tensor Imaging (DTI), Dynamic Susceptibility Contrast (DSC)-MRI, Dynamic Contrast Enhanced (DCE)-MRI, MR Spectroscopy (MRS)) have expanded the possibility of preoperative tumor

heterogeneity evaluation. The integration of these techniques into texture analysis and machine learning techniques has significantly advanced the field of radiogenomics. This progress holds great potential for identifying intratumoral subpopulations with distinct genetic or microenvironmental characteristics [46–52] and supports the preoperative discrimination of glioma grades [53–55]. Furthermore, radiomics addresses other relevant clinical questions, such as the assessment of tumor infiltration beyond the contrast-enhancing tumor and the accurate differentiation between tumor progression and treatment effect (pseudoprogression) [56–59].

1.4. Neuropathology

The diagnosis of GBM primarily relies on the histopathology assessment of formalin-fixed, paraffin-embedded tissue from a resected or biopsied tumor **(Figure 2)**. Molecular profiles of GBM were introduced in the 2016 WHO classification of CNS with the concept of IDH-mutated and IDH wild-type GBM. At that time, three IDH wild-type GBM morphological variants were considered: giant cell GBM, gliosarcoma and epithelioid GBM [60]. In 2021, the WHO classification of CNS tumors further refined the molecular-based diagnostic criteria. For instance, in tumors with typical histologic features of GBM, the presence of *IDH* mutation re-classifies these tumors as astrocytomas, IDH-positive, grade 4. In the absence of typical GBM histologic features, the diagnosis of GBM IDH-wild-type is based on the presence of specific molecular alterations, including gain of chromosome 7, loss of chromosome 10, *TERT* promoter mutations and *EGFR* amplification. For IDH-positive astrocytomas, *CDKN2A/2B* homozygous deletion confers prognostic value [61].

1.4.1. Special GBM phenotypes

The microscopic appearance of GBM is a typical infiltrative tumor with predominant astrocytic morphology, marked pleomorphism, brisk mitotic activity, microvascular proliferation and rich necrotic areas. As expected by its previous denomination, Glioblastoma Multiforme, a variety of phenotypes are frequently observed within the same tumor. As mentioned, previous WHO classifications included some of these morphological variants as entities, as they were commonly associated with distinct MRI characteristics and patient prognosis [61].

Accounting for 2-8% of all GBM cases, gliosarcoma (GS) displays typical GBM morphological features combined with areas with sarcomatous or mesenchymal differentiation, commonly resembling a fibrosarcoma. Infrequently, lipomatous, osteoid, chondral or osteochondral elements may also be observed [60,62–65]. The molecular features of GS show significant overlap with those of GBM and soft tissue sarcoma, although a genomic signature includes frequent alterations in the *TERT* promoter, *PTEN*, *TP53*, *NF1*, *CDKN2A*/*B*, *RB1* and *STAG2* genes has been proposed [63,65–68]. Despite being first described in 1897 as a GBM-variant, GS remains poorly characterized, with limited understanding of its distinct clinical and imaging characteristics, as well as response to treatment. Published series point to a tendency for cortical involvement, often in the temporal lobe, with frequent dural contact, higher proportion of enhancing areas and low necrosis proportion [69,70]. A higher propensity for extracranial metastasis has been also described [65,71]. Retrospective studies on GS report a median survival ranging from 8.3 to 16.7 months. However, GS is generally perceived to have a poorer prognosis compared to GBM, even with the implementation of optimal treatment strategies [63,65,69,72,73].

Epithelioid GBM (Ep-GBM) is a rare morphological GBM variant (2-3%), characterized by closely packed large epithelial-like cells, with abundant eosinophilic cytoplasm and prominent nucleoli [60,74]. They are frequently associated with *BRAF* V600E mutations, *TERT* promoter mutations and homozygous deletion of *CDKN2A*/*2B* [75–78]. DNA methylation-based analysis suggests

that epithelioid GBM can be stratified into three distinct groups: pleomorphic xanthoastrocytoma-like tumors with a favorable prognosis, IDH wild-type GBM-like tumors with a poor prognosis, and an intermediate RTK I pediatric GBM-like tumors [79,80]. Clinical and MRI characterization of the epithelial variant relies on case reports and small series. Ep-GBM seems to occur in younger patients and has been described to convey an aggressive behavior, with frequent cerebral spinal fluid (CSF) dissemination [78,81]. Mainly located in the cerebral lobes, it presents most often as a single, well-circumscribed, superficial lesion [82–84]. Curiously, the presence of calcifications has been described in this type of tumor [84].

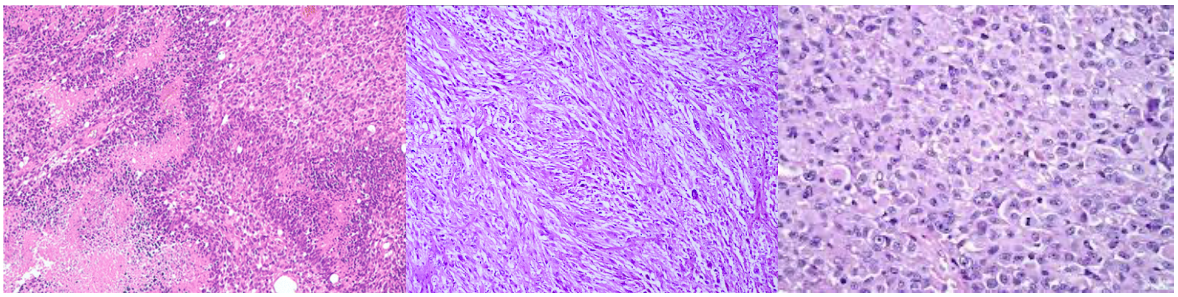

Figure 2. Phenotypic diversity in Glioblastoma. Examples of hematoxylin & eosin staining in GBM in which it is noticeable the phenotypic diversity associated with this tumor. A typical GBM with pseudopalisade necrosis; a gliosarcoma with mesenchymal dedifferentiation and epithelioid GBM with epithelial-like differentiation. Data retrieved from ULS Santo Antonio (DEFI104-17 (093-DEFI/091-CES)

1.4.2. GBM molecular features and tumor heterogeneity

In GBM, contrary to lung cancers or melanoma, critical molecular and chromosomal alterations occur early in molecular time [15]. Loss of chromosome 10 and driver mutations in *TP53* and *EGFR* genes are early events, while mutations in the *TERT* promoter tend to occur at early to intermediate time points. Indeed, 90% of GBM were shown to consistently contain alterations within the first 10% of the molecular time, which suggests that they arise very early in a patient's lifetime [85].

GBM was the first tumor type analyzed by The Cancer Genome Atlas (TCGA) project; a landmark large-scale sequencing effort aimed at characterizing its genomic landscape. Defects in three major pathways central to GBM biology were identified: the p53/stress response pathway, the Rb/cell cycle control pathway, and the receptor tyrosine kinase (TKR)/ Ras/phosphoinositide 3-kinase (PI3K) signal transduction pathway [86]. While these findings highlighted the involvement and convergence into well-established cancer pathways, they also revealed extensive genomic intertumor heterogeneity. Through consensus clustering analysis, four expression-based GBM subgroups were established: proneural (characterized by frequent *PDGFRA*, *SOX2*, and *MYC* amplifications, as well as mutations in *IDH1*, *TP53*, and *ATRX*), mesenchymal (associated with *NF1* mutations), classical (marked by *EGFR* alterations and *CCNE1* amplification), and neural (defined by neuronal markers expression) [87,88]. Beyond this well-document intertumoral heterogeneity, subsequent research has emphasized the substantial intratumoral heterogeneity present in GBM. For instance, spatial analysis of GBM proteome showed multiple signatures within the same tumor, which had been previously individually associated with different survival characteristics [89,90]. Longitudinal genomic and transcriptomic analysis suggest that the majority of GBM undergoes expression-based subtype changes, while estimates of evolutionary rate suggest that relapse-associated clones exist long before diagnosis [91]. Most importantly, intratumoral heterogeneity encompasses differences in the tumor microenvironment, which interacts dynamically and reciprocally influences genetic and epigenetic cellular states [92].

Collectively, these findings suggest that GBM survival and response to therapy are likely determined by the interplay of diverse tumor subclones. This complexity largely accounts for the disappointing results of targeted therapies in GBM, as treatment-resistant subclonal populations are often selectively enriched.

1.5. Treatment of primary GBM

The standard of care for GBM is multimodal treatment with a combination of surgery/biopsy followed by adjuvant chemotherapy and radiotherapy [3,93]. The primary goal for surgery or biopsy is to obtain tissue for a histopathological and molecular diagnosis. Over the last decades, many studies have assessed the association between extent of resection and survival outcomes in GBM. Collectively, the evidence consistently demonstrates a survival benefit with greater extents of resection and lower residual volumes [94,95]. Preservation of the neurological functions remains a high priority and, thus, maximal safe resection is currently recommended by all major American and European Oncology and Neurosurgical societies [3,93].

Following surgery, six weeks of radiotherapy with concomitant temozolomide and subsequent six cycles of adjuvant temozolomide became the standard treatment for newly diagnosed adult GBM patients, after an improvement in patient overall survival and absolute increases in 2 and 5-year survival rates was established by the randomized clinical trial EORTC 26981-22981/NCIC CTG CE.3 [96]. Nevertheless, the application of these results to specific populations, such as the elderly or frail patients, is more questionable and other treatment schemes have been proposed (reviewed by [27,97,98]).

The limited treatment options for GBM, especially when compared to other cancers, stem from its intrinsic characteristics and inevitably result in persistently poor survival outcomes. Beyond its diffuse growth and infiltrative nature, three key features have significantly hindered the effective application of treatments to GBM. First, the physical obstacle presented by the blood brain barrier (BBB) to the delivery of systemic treatments. This underlies the rationale for local treatment of GBM, for instance with BCNU (carmustine) wafers. Although initial studies in both primary and recurrent GBM suggested that the use of BCNU wafers was safe and associated with a survival benefit, the limited clinical benefit and occurrence of important adverse events, removed BCNU wafers from treatment

recommendations in GBM [99,100]. Other strategies for local delivery are currently in development and include convection-enhanced delivery methods or the use of implanted reservoirs in the tumor for drug delivery(reviewed by [101,102]).

Then, another important issue is the lack of relevant molecular targets for GBM. Clinical trials dedicated to targeted-therapies for GBM have thus far largely resulted in failure. A critical limitation to the efficacy of these therapies is their limited consideration for GBM unique biology, including its intra and intertumoral heterogeneity and redundant signaling pathways [88,103]. Current areas of clinical research for targeted therapy in GBM include *EGFR* amplification or *EGFRvIII* mutation [104,105], TERT promoter mutations [106], *FGFR* mutations [107], *BRAF* V600E mutation [108,109], PI3K/AKT/mTOR pathway [110–112] and RB pathway [113].

Finally, GBM is an exceptionally complex and profoundly heterogeneous tumor, with a critical gap in the availability of reliable disease biomarkers. This limitation significantly hinders the identification of clinically relevant subgroups capable of consistently predicting treatment response.

1.5.1. Immune Checkpoint inhibitors

There are several challenges to the development of effective immunotherapies in GBM, a field that has brought a significant improvement in patients' outcome in various systemic cancers. These include the CNS immunologic privilege, GBM-induced severe local and systemic immunosuppression, its poor immunogenicity and the frequent corticosteroids usage for symptomatic control.

Immune checkpoint inhibitors tested in GBM include anti-programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In newly diagnosed GBM, no benefit was observed for the addition of

nivolumab to radiation therapy, when compared to temozolomide plus radiation therapy (CheckMate-498, NCT02667587) [114,115]; or for the addition of nivolumab to temozolomide and radiation therapy in methylguanine-DNA methyltransferase (MGMT)-methylated patients (CheckMate-548, NCT02617589) [116]. For recurrent GBM, nivolumab failed to be superior to bevacizumab in another clinical trial (CheckMate-143, NCT0201771), with a response rate of 7.8% to nivolumab compared to the 23.1% response to bevacizumab [117]. Notwithstanding, the median response for nivolumab was 11.1 months, noticeable higher than the 5.3 months median response to bevacizumab. One promising result with a significant improvement in overall survival was observed using immune checkpoint inhibition with pembrolizumab in the neoadjuvant setting [118]. Although mostly disappointing, these results reiterate the importance of identifying biomarkers to ensure proper patient selection in GBM clinical trials.

In addition to treatment with immune checkpoint inhibitors, other immunotherapies explored in gliomas include neoantigen vaccines (ACT IV study, a peptide vaccine targeting EGFRvIII) [119], personalized neoantigens derived from individual GBM transcriptomes and immunopeptidomes (NCT04015700, NCT02287428) [120], viral therapies with oncolytic viruses (engineered poliovirus (PVSRIPO, NCT02986178) [121], or gene therapy (engineered retrovirus (Toca 511, NCT02414165)[122] or adenovirus (NCT00589875) [123], as a means to induce immune surveillance. In addition, the use of engineered chimeric antigen receptor (CAR) T-cells is being increasingly explored [124–126].

1.6. Recurrent Glioblastoma

Despite important advances in surgery and tumor-directed treatment, tumor recurrence in GBM is inevitable. GBM recurrences are mostly local, within 2 cm of the initial margin [127,128]. Some cases have distant recurrences, reported to occur in 2 to 34.5% of the cases [129–134]. Distant recurrences appear to portend a worse prognosis, although its biology is not

completely clarified. Factors suggested to underly distant tumor recurrence include MGMT promoter methylation [135,136] and involvement of the subventricular zone (SVZ) [137,138].

Further studies have focused on the genetic landscape of the recurrent tumor cells, based on the analysis of matched primary-recurrent tumors. Overall, these studies propose a highly branched evolutionary pattern for GBM recurrence, indicating that recurrent tumor cells frequently arise from a different dominant clone. This pattern likely reflects the alternative evolutionary paths that confer treatment resistance [91,139–141]. Moreover, one study reported that distant recurrences shared a lower proportion of mutations with the primary tumor (25% vs 70% for local recurrences), thus suggesting that distant recurrences may originate from the primary tumor at an earlier evolutionary stage [142].

1.7. Patient survival and prognostic factors

According to the Central Brain Tumor Registry of the United States (CBTRUS), the 5-year survival rate for GBM was limited to 6.9% between 2015-2019 [1] and the median survival is only 8 months. Reported median overall survival in clinical trials and surgical series usually ranges between 10-18.1 months [117,143–145].

Minimal progress in overall survival has been observed over the last decades for GBM patients, contrary to what is documented for other malignant tumors, including primary central nervous system tumors as medulloblastoma, as a response to advances in systemic therapies [146,147]. Nevertheless, clinical series comparing patient survival before and after the implementation of the trimodal treatment as proposed by Stupp *et al*. in 2005, have consistently demonstrated the positive impact of this regimen [148].

In order to guide treatment decision, the impact of several tumor characteristics and different patient managements on survival has been studied for GBM. Recently, molecular

characteristics have become prominent prognostic factors, in particular MGMT promoter methylation status [149,150] and IDH mutations [151,152].

Apart from tumor molecular characteristics, other established prognostic factors include clinical characteristics, such as younger age and better performance status [153–156], as well as extent of tumor resection [95] and amount of residual contrast-enhancing tumor [157]. Although with disputable results, several characteristics have correlated with patient survival, including clinical presentation [33,35,158], tumor location [153,159], multifocal disease [41,160], initial lymphopenia [161], high neutrophil-to-lymphocyte ratio (NLR) [162,163] and other systemic inflammatory scores [164].

GBM patients surviving over 36 months are referred to as long-term survivors. The largest series characterizing these patients showed a median overall survival of 4.6 years, ranging from 3 to 15.3 years [165]. A systematic review showed a doubling of 2- and 3-year survival rate since the introduction of Stupp protocol in 2005 [166]. Nevertheless, the pooled 5-year survival estimates were 3% for periods earlier than 2005 and only 5% for later periods, confirming a poor improvement for longer survival in GBM [166]. Characteristics associated with long-term survival in GBM have been inconsistently described and include young age [167–172], better performance status [167–169], tumors with IDH mutation [172,173] and MGMT methylation [165,170–173]. It has been also described gene enrichment for sphingomyelin metabolism in these tumors [170].

2. The Immune System and Glioblastoma

The concept of a static and inert immunological environment within the CNS has been gradually challenged by groundbreaking discoveries in the last decades. The description of structures that resemble conventional lymphatic vessels paralleling the dural venous sinuses [174], as well as evidence that antigen-presenting cells could travel from the CNS to cervical lymph nodes [175], helped to understand multiple sclerosis as a T-cell driven disease [176,177] or the CNS manifestations in paraneoplastic auto-immune reactions for non-CNS tumors [178]. The brain has an immunological privilege, being dynamically regulated with exchange of cellular and molecular cues during various processes, such as infection and inflammation [179]. This paradigm shift motivated efforts to understand the role of immune cells in gliomas.

2.1. Immunosuppression and inflammation in Glioblastoma

Cancers originate from healthy tissues; however, the mutations underlying malignancy create a unique molecular identity for cancer cells. These tumor-specific neoantigens prompt immune recognition, resulting in the clearance of cancer cells before they consolidate into a solid tumor. Unfortunately, malignant cells can hijack the protective mechanisms that safeguard healthy tissues against autoimmune reactions or uncontrolled inflammation. Consequently, cancer cells are able to evade detection, and hence destruction, by the immune system [180].

GBM is a notoriously immunosuppressive tumor, capable of inducing a profound immunosuppressive environment through a multitude of mechanisms. The resulting tumor microenvironment harbors abundant immunosuppressive cells, including tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and regulatory T cells (Tregs). This environment exhibits strong modulation of cell surface molecules and an overactive secretion of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming

growth factor-ß (TGFß) [181–183]. Altogether, these factors diminish the recruitment and infiltration of effector T cells, ultimately leading to dysfunction in effector T cell responses [184–186]. In addition to creating a profoundly local immunosuppressive environment, GBM has the capacity to induce severe systemic immunosuppression, as evidenced by impaired cellular immunity in patients [187].

Similarly, several types of chronic inflammation have been causally associated to cancer development and progression, as demonstrated by gastric cancer and MALT (mucosa-associated lymphoid tissue) lymphoma association to the persistent Helicobacter pylori infection [188]. The inflammatory response is triggered as a normal host defense for pathogen elimination, which is then subverted by the tumor. Even in the absence of pathogens, solid tumors can exploit the intrinsic inflammatory response and create a pro-tumorigenic microenvironment [189], with the ability to promote continuous cell renewal and proliferation, activate tumor-promoting transcriptional programs, cause oncogenic mutations and genomic instability, and trigger the angiogenic switch [188,190,191].

The GBM microenvironment represents a highly heterogeneous and dynamic system, hosting neoplastic cells alongside resident non-neoplastic cells and a diverse composition of infiltrating and resident immune cells. Recent evidence supports that the cellular composition of GBM microenvironment varies according to its subtype and the characteristics of the individual patient [185,192–195], and latest studies have convincingly associated tumor-mediated immunosuppression with poor patient prognosis [181,183,196,197]. It is important to consider the effects of current GBM treatments, a standard regimen of chemotherapy and radiotherapy, as well as the high doses of corticosteroids frequently employed for symptomatic control of brain edema. Unfortunately, chemoradiation protocols trigger strong inflammatory responses associated to the massive necrosis induced[198], and the combination of all these therapies further contribute to patient immunosuppression, with a significant impact in GBM patient survival [161,199–201].

The systemic impact of the local immunosuppression and inflammation caused by

malignancies is reflected by hematological indicators, which demonstrate prognostic prediction in various tumors, including esophageal squamous carcinoma, pulmonary neuroendocrine carcinoma, hepatocellular carcinoma, nasopharyngeal, non-small cell and adenocarcinoma lung cancer [202–207]. Similarly, hematological factors have been extensively studied in the last decades for prognosis prediction in GBM. One such marker of systemic inflammation, neutrophil-to-lymphocyte ratio (NLR) is an easily accessible parameter, which has been highly validated in GBM cohorts. An elevated NLR in peripheral blood predicts a poor patient prognosis [208–211], independently of MGMT status [210].

2.1.1. T-cell dysfunction

Robust evidence has highlighted the pivotal role of T-cell dysfunction in GBM-associated immunosuppression, with important implications for tumor progression, aggressiveness and recurrence. All five domains of T-cell dysfunction have been implicated in GBM: exhaustion, ignorance, senescence, tolerance and anergy. T-cell exhaustion refers to the repeated exposure of T cells to antigen under suboptimal conditions, as observed in chronic viral infections. GBM induces a similar phenotype of T-cell exhaustion, with activation of specific transcriptional programs leading to hyporesponsive T cells[212]. In addition, expression of immune checkpoints (ICP), such as PD-L1, is induced in the exhausted state [212–214] T-cell exhaustion is particularly severe in GBM comparing to other types of cancers, frequently with expression of various ICP[212]. Other dysfunction domain, T-cell ignorance is the result of inadequate antigen presentation to functional T cells, due to anatomical barriers or low levels of antigen expression. In the particular case of GBM, the existence of a BBB combined with the low quantity of T cells in circulation prevents the immune system from making robust T-cell mediated responses. An interesting study proposed, for the first time, blood marrow T cell sequestration as a mechanism for T-cell dysfunction specifically in GBM [215]. This contributes to T-cell ignorance and explains the profound lymphopenia observed in some

GBM patients upon initial evaluation [200].

Reports on tumor infiltrating lymphocyte (TIL) in GBM show significant variability regarding incidence, degree of infiltration and spatial distribution within the tumor [216,217]. Recently, GBM subtype-specific immune profiles have been proposed, which can partly explain these discrepancies [192–194,218,219].

Most important, various reports document TIL infiltration most commonly in perivascular areas rather than tumor parenchyma, thus suggesting an inability of TIL to effectively migrate into GBM immunosuppressive microenvironment [217,218].

2.1.2. Immune-checkpoint molecules

(a) Mechanisms

Immune checkpoints (ICP) are critical regulators of the immune system, with the important role of ensuring that immune responses are appropriately targeted and controlled. They guarantee that antigens from pathogens (non-self) are identified and eliminated, while preventing excessive immune activation, as observed in autoimmune diseases. Two signals are required for an immune T cell to eliminate a non-self cell: the first signal is the recognition of an antigen presented on the surface of the cell by a T cell, while the second signal is a co-stimulatory molecule that activates the immune cell. In the absence of the second signal, it is inferred that the cell is part of normal tissue (self) and should not be destroyed **(Figure 3)**. Several ICP have been described, including PD-1 (Programmed cell-death Protein-1), CTLA-4 (Cytotoxic T-Lymphocyte-Associated Protein 4), TIM3 (T-cell Immunoglobulin and Mucin Domain-Containing Protein 3), LAG3 (Lymphocyte Activation Gene 3), BTLA (B- and T-Lymphocyte Attenuator), 2B4 (Natural Killer Cell Receptor 2B4), SLAMF6 (Signaling Lymphocytic Activation Molecule Family member 6), TIGIT (T-cell Immunoreceptor with Ig and ITIM domains) and CD39 [220,221].

CTLA-4 was the first immune checkpoint discovered and it is similar to the co-stimulatory molecule CD28. CTLA-4 competes with CD28 for binding to CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells (APC), and ultimately inhibits T cells [222]. Gradually, other immune checkpoints were discovered and subsequent research unraveled the intricate nuances of these immunological mediators, far surpassing the simplistic notion of binary controls. Each immune checkpoint has distinct functions and specificities, being able to signal either alone or in coordination with others. For example, CTLA-4 is up-regulated upon initial T cell activation in lymph nodes, while PD-1 signaling occurs primarily within peripheral tissues or within the tumor microenvironment. In addition, PD-1 binds at least two biological relevant molecules, PD-L1 (Programmed Death-Ligand 2) and PD-L2 (Programmed Death-ligand 2), with different locations and expression patterns [223].

After the initial description of PD-1 in 2000 [224], preclinical studies have confirmed the anti-cancer benefit of blocking either PD-1 or PD-L1 [225,226]. Indeed, in the last few years, CTLA-4, PD-1 and PD-L1 inhibition have consistently exhibited activity with survival benefit in melanoma and epithelial cancers [227,228] **(Figure 3)**. Biomarkers of immunotherapeutic benefit for PD-1/PD-L1 inhibition include higher somatic tumor mutation burden [229–231] and PD-L1 expression [232]. In addition, overexpression of PD-L1 associates with worse overall prognosis in various solid tumors, including breast and ovarian cancer [233–236].

(b) PD-L1 expression in GBM preclinical and clinical studies

Clinical successes in other solid malignancies [220], motivated the examination of immune checkpoint blockade in GBM. Preclinical models quickly provided compelling evidence to support the idea of ICP blockade in this tumor [237]. As an example, the combination of anti-PD-1 blockade with stereotactic radiation led to long-term survival in mice with intracranial gliomas [238]. Blockade of the PD-1 and/or CTLA-4 pathways was able to eradicate established GBM in the majority of treated mice, while inducing a protective immune memory that prevented tumor recurrence upon rechallenge [239].

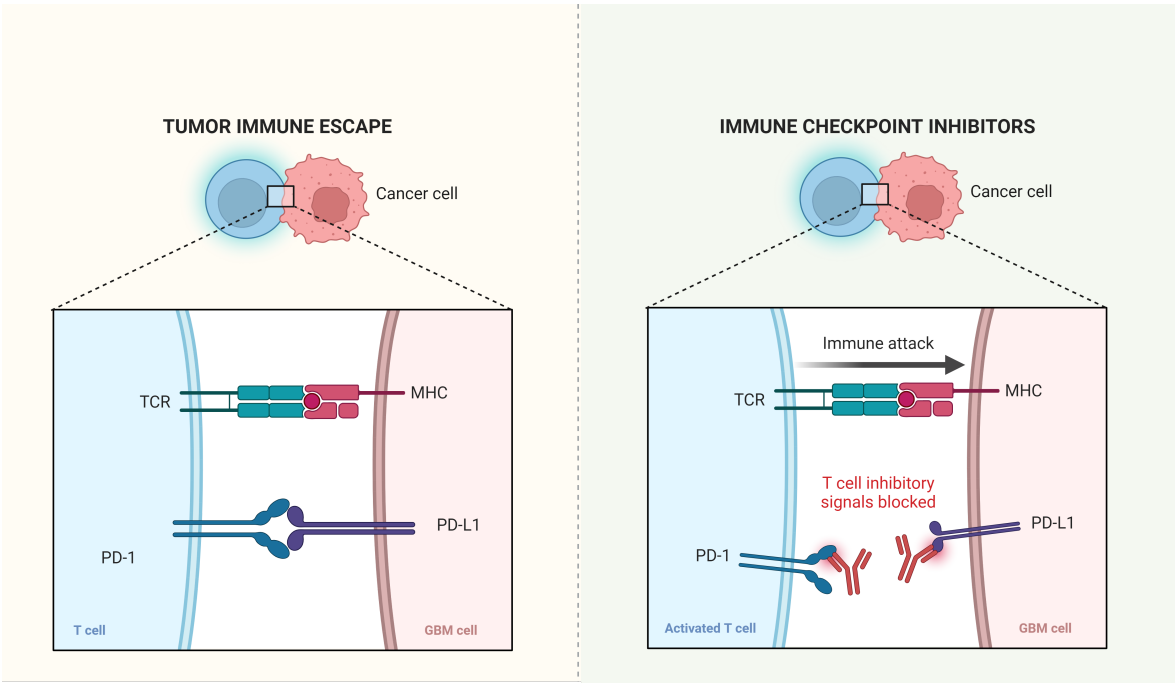

Figure 3. PD-1/PD-L1 interactions and its blockade using PD-L1 or PD-1 inhibitors. MHC molecules present antigens on their surface to T cells, which allows them to recognize and respond to these antigens. PD-L1 expressed on tumor cells binds PD-1 on T cells, sending an inhibitory signal to suppress the immune response. Blocking this interaction with monoclonal antibodies (immune checkpoint inhibitors) restores T cell activity, enabling the immune system to attack cancer cells effectively. Created with Biorender.

PD-L1 positivity rates in GBM patient cohorts vary widely across studies **(Table I)**. Some reports indicate high PD-L1 expression rates (>85%), suggesting a prominent role for PD-1/PD-L1 axis in GBM-mediated Immunosuppression [214,217,240,241]. However, the reported range of PD-L1 positivity in the literature spans from 5.7% to 100%. A pooled analysis estimates an overall positive percentage of 44.72% [242]. Several factors have been associated with PD-L1 expression in GBM, namely TIL infiltration [217,243], IDH wild-type status [219,244,245] and the mesenchymal subtype [217,243,244]. Notably, differences between PD-L1 expression in primary and match-recurrent tumors appear to be minimal [217,246,247]. Finally, and similarly to other cancers, PD-L1 expression has been consistently associated with worse overall survival in various studies [214,246,248–250] , including two meta-analysis [251,252].

Table I. PD-L1 expression in Glioblastoma patient series.

Author/year	Expression rate of PD-L1 in GBM	Other results	Prognosis
Wintterle *et al*. 2003	100% (9/9 specimens)		
Wilmotte *et al*. 2005	100% (19/19 specimens)	Higher PD-L1 positive expression in GBM samples	
Liu *et al*. 2013	17 GBM specimens		Worse patient survival
Berghoff *et al*. 2015	Membranous expression 37.6% (44/117 newly diagnosed) and 16.7% (3/18 recurrent) Diffuse/fibrillary expression 84.6% (99/117 newly diagnosed) and 72.2% (13/18 of recurrent GBM)	Higher PD-L1 associated with mesenchymal subtype	No significant correlation
Zeng *et al*. 2016	68.6% of grade IV gliomas (229 specimens)		No significant correlation
Nduom *et al*. 2016	60.6% of the specimens with >1% PD-L1 expression; 38.3% of the specimens with >5% PD-L1 expression (99 specimens)		A trend toward decreased survival
Xiu *et al*. 2016	19% (1035 specimens)		
Garber *et al*. 2016	35% (57/163 specimens)	Higher expression according to WHO grade	
Berghoff *et al*. 2017	Membranous expression 56.2% (68/121) of IDH-wild type glioma and 5.7% (3/53) of IDH-mutant glioma. Diffuse/fibrillary expression 84.3% (102/121) of IDH wild-type glioma and 37.3%(20/53) of IDH-mutant glioma.	Higher expression in IDH wild-type gliomas	
Heiland *et al*. 2017	48 GBM specimens.	Higher expression in IDH wild-type GBM and the mesenchymal subgroup.	
Xue *et al*. 2017	87.8% (41 specimens)	PD-L1 levels correlated with WHO grade, VEGF status and ki-67.	
Hodges *et al*. 2017	10.1% (189 specimens)	Low expression in IDH-mutant glioma	
Miyazaki *et al*. 2017	16 GBM specimens	No differences in matched primary-recurrent tumors	No significant correlation
Han *et al*.2017	31.5% (54 specimens)		Worse patient survival
Heynckes *et al*. 2017	GBM 20.8% (primary GBM) and 7.6% (matched-recurrences)		
Zhang *et al*. 2017	59.7% (37/62 brainstem glioma specimens)		
Lee *et al*.2018	32.2% (115 specimens)	High PD-L1 expression in IDH wild-type	Worse patient survival

(c) Clinical trials targeting the PD1/PDL1 axis

Tumor immunotherapy is a therapeutic approach which aims to reactivate a tumor-specific immune response and thereby the T cell capacity to control and eliminate tumors cells. To date, several immune-stimulatory therapies have been proposed for GBM and many of these have been tested in clinical trials. However, despite the promising results from preclinical studies, immunotherapy has yet to demonstrate a convincing benefit in median survival for GBM patients. Many GBM characteristics are to be considered for this setback. GBM is considered a "cold tumor" and its low immunogenicity predicts a worse response to immunotherapy. Another particularity of GBM is its location in the unique immunologic privilege of the CNS, with an intrinsic regulatory activity. Finally, the profound local and systemic immunosuppression exerted by GBM, to a degree infrequently observed in other tumors, also limits the clinical benefits of immunotherapy (reviewed by [257]). Indeed, for immune checkpoint blockade to be effective, a baseline immune response must be present.

Explored molecules include:

- Nivolumab (NIVO), a human IgG4 monoclonal antibody that targets PD-1 by binding to the PD-1 receptor, thereby blocking its inhibitory effect. In GBM, it has been combined with ipilimumab, which targets CTLA-4, for the CheckMate 143 trial (NCT02017717). Other clinical trials combined nivolumab with current GBM treatments in MGMT methylated (NCT02667587) or unmethylated (NCT02617589) patients.
- Pembrolizumab (Pembro), also targets PD-1 and is a humanized antibody. It was evaluated in the neoadjuvant setting in a clinical trial [118], and was found to be more effective than adjuvant alone. In addition, an increase of intratumoral T cells was clearly observed [118].
- Durvalumab is a high-affinity IgG1 monoclonal antibody that blocks the binding of PD-L1 to PD-1. Initially evaluated for recurrent GBM, it showed promising results regarding 6-month progression-free survival rate [258] and s currently being evaluated in combination with standard radiotherapy in newly diagnosed unmethylated patients [259].

- Avelumab monoclonal antibody targets PD-L1 and is currently being evaluated in several Phase I and Phase II trials, including one evaluating the safety and tolerability of treatment combination of avelumab with laser interstitial thermal therapy (LITT) (NCT03341806).

Clinical trials with PD-1/PD-L1 axis blockade are resumed in **Table II**.

Table II. Clinical Trials in PD-1/PD-L1 axis blockade in GBM.

Clinical Trial / Phase		Treatment	Indication	Results	Immunological response
CheckMate 143 NCT02017717	III	NIVO vs bevacizumab and NIVO ±ipilimumab	Recurrent GBM	No impact OS=9.8 vs 10.0	Did not meet primary endpoint
CheckMate 498 NCT02617589	III	NIVO + RT vs TMZ + RT	Recurrent GBM requiring surgery	No impact OS= 13.4 vs 14,9 months PFS=6.0 vs 6.2 months	Did not meet primary endpoint
CheckMate 548 NCT02667587	III	NIVO + QT/RT	MGMT-methylated GBM	No impact PFS=10,6 vs 10,3 months OS=28,9 vs 32,1 months	Did not meet primary endpoint
MK-3475 NCT02337491	II	Pembro + bevacizumab vs Pembro	Recurrent GBM	No impact PFS=4.1 vs 1.4 OS=8.8 vs 10.3 months	Did not meet primary endpoint
NeoNivo NCT02550249	II	NIVO (neoadjuvant) + surgery + NIVO (adjuvant)	Recurrent GBM requiring surgery	Survival benefit 7.3 months	Increased chemokine transcript expression, Immune cell infiltration, TCR clonal diversity in tumor.
MK-3475 NCT02852655	I	Pembro (neoad), surgery, Pembro (ad)	Recurrent GBM requiring surgery	13.7 months vs. 7.5 months	Pre-surgical ICB enables a selective, primary tumor-specific T cell clonal modulation.
NCT02337686	II	Pem (neoad), surgery, Pem (ad)	Recurrent GBM requiring surgery	PFS= 4,5 months OS=20 months	Rare CD8+ T cells and abundant CD68+ Macrophages in GBM tissue.
NCT02336165	II	Durvalumab + RT	Recurrent GBM requiring surgery	OS= 15.1 months	
GliAVax NCT03291314	II	Avelumab + axitinib	Recurrent GBM	No impact PFS 6 months 18.5% vs 22.2% OS 18.0 vs 26.6 weeks	
NCT03047473	II	Avelumab + QT/RT	GBM	PFS= 9.7 OS=15.3 months	
NCT04396860	II	Ipilimumab (Ipi) + NIVO + RT vs QT/RT	MGMT unmethylated GBM	To be posted	

3. Epithelial-to-Mesenchymal transition in Glioblastoma

Epithelial-to-mesenchymal transition (EMT), originally described as part of early embryogenesis, is a well-defined cellular program set by changes in gene expression and post-translational regulation mechanisms, in which epithelial characteristics are down-regulated and a mesenchymal behavior and phenotype is acquired [260]. It is well established that EMT includes a variety of morphogenetic events, which enables the detachment of epithelial cells from their epithelial sheet and the acquisition of a mesenchymal state which accounts for an increase cell plasticity, migration and invasion capacity [261].

During embryo development, epithelial cells undergoing EMT are morphologically diverse and range from the primitive cells giving rise to mesoendoderm, with incompletely assembled cell-cell junctions, to cells with fully formed cell-cell junctions, including adherens junctions [262]. Indeed, most adult tissues arise from a series of conversions from epithelial to mesenchymal cells, through EMT and the reverse process, MET (mesenchymal-to-epithelial transition). These sequential rounds of conversions in development are referred to as primary (implantation, gastrulation and delamination of neural crest cells), secondary (notochord, the somites, somatopleure, splanchnopleure) and tertiary (cushion mesenchyme) EMT [263]. Other cellular processes, as the physiological response to injury, also parallel EMT cellular programs [264,265]. The EMT program is therefore divided into three functionally different subtypes, depending on the physiological context: type I, in embryo development; type II, in wound healing, tissue regeneration and fibrosis; and type III, in tumor development [263,266,267].

EMT activation triggers several cellular processes that ultimately combine in a stable and long-lasting, albeit reversible, cell phenotypic and mechanistic regulation. Several types of epigenetic modifications have been described in EMT, resulting in a highly modified chromatin landscape according to cell requirements [268–270]. The classical transcription factors (TF) associated to EMT can be divided into three families: SNAIL (SNAIL1 and SNAIL2),

ZEB (ZEB1 and ZEB2) and TWIST (TWIST1 and TWIST2) [271]. These factors are a requisite for EMT programs in specific cellular contexts, working mostly as transcriptional repressors of epithelial genes [272–274]. Consequently, a central result in EMT is the loss of E-cadherin expression, which maintains cell-cell adhesion. As such, regardless of the mechanism, direct binding to the E-cadherin promoter or indirect repression of E-cadherin transcription, the vast majority of the signaling pathways known to trigger EMT converge in the induction of the classical EMT-TF which are E-cadherin repressors [275,276]. In addition to repressing epithelial cell polarity and promoting a mesenchymal state, they also suppress cell division, which favors invasion and promotes cell survival, conferring advantage to migratory cells [275,277]. In many cases, a quasi-mesenchymal state is reached through changes in the nature and dynamics of the junctions, rather than the complete loss of cell-cell junctions [278,279]. Likewise, the transition from an epithelial to a mesenchymal state is often incomplete, resulting in cells in an intermediate state with a combination of epithelial and mesenchymal characteristics. These intermediate states are diverse, dependent on the biological context, and most likely reflect the multiple alternative paths and insults that trigger the advance towards a mesenchymal state [277]. For instances, in wound healing, cells activating EMT programs frequently express combinations of epithelial and mesenchymal markers, suggesting that these intermediate states, referred to as epithelial-mesenchymal plasticity (EMP), are frequent in physiological states and thus indispensable [280].

3.1. EMT in cancer

EMT in cancer is frequently simplified as the acquisition of mesenchymal features that enable tumor invasion and metastasis. It is, in fact, as previously introduced, a highly diverse, transitory and meticulously regulated dynamic program. Although initial evidence of the EMT in cancer emerged from studies on the well-characterized epithelial carcinoma context,

compelling data has demonstrated its involvement in the pathology of other non-epithelial tumors, highlighting the broad phenotypic spectrum in which EMT orchestrates [281].

Current knowledge supports that EMT-dependent and independent invasion occurs simultaneously at the tumor invasion front [282] and that pathological EMT may occur as various focal events within a tumor [283]. Similarly to embryonic EMT programs, cancer-associated activation of EMT impinges not only on alterations on cell morphology, invasion and motility, but also on the acquisition of stem cell-like properties. Indeed, studies have demonstrated that EMT activation occurs at the periphery of human embryonic stem cells, in parallel to the expression of several pluripotent transcription factors [284,285]. This occurs also in cancer stem cells (CSC), where the induction of the mesenchymal phenotype may enhance stemness preservation and cell migratory properties [286–289]. Furthermore, the ability for self-renewal and to reconstitute all the lineages of the tissue of origin, allows CSC the ability to recreate both epithelial and mesenchymal lineages [286,290]. Within a tumor, there are both CSC and somatic tumor cells exhibiting EMT activation, which explains why some cells that undergo complete EMT become fixed in the mesenchymal lineage; while, in the same tumor, another population has higher plasticity and can undergo the reverse process through MET and constitute the metastatic distant niche with an epithelial differentiation [288]. Akin to the EMT in physiological states, the concept of EMT in cancer is increasingly regarded as a complex non-binary and dynamic program, with frequent intermediate states **(Figure 4)**. In the EMP, often referred to partial, mixed or hybrid EMT, phenotypical and functional changes appear to be regulated as a continuum [291]. A recent study has identified and characterized the presence of multiple unique intermediate EMT states and two distinct mesenchymal-like states within the same tumor [292]. Strikingly, they observed that although they identified subclones with higher plasticity, migratory and invasion potential, no individual subclone is able to recapitulate the tumor aggressiveness of the parental line. The EMP unquestionably contributes to the complex tumor heterogeneity [291,293] and perhaps is the heterogeneity of the various subpopulations, while maintaining a

small subpopulation with higher plasticity, that confers the highest advantage to the tumor. Taken together, focal and partial EMT may underly the incongruous results in the literature, and thus setbacks in the evaluation of the relevance and mechanisms of EMT in cancer.

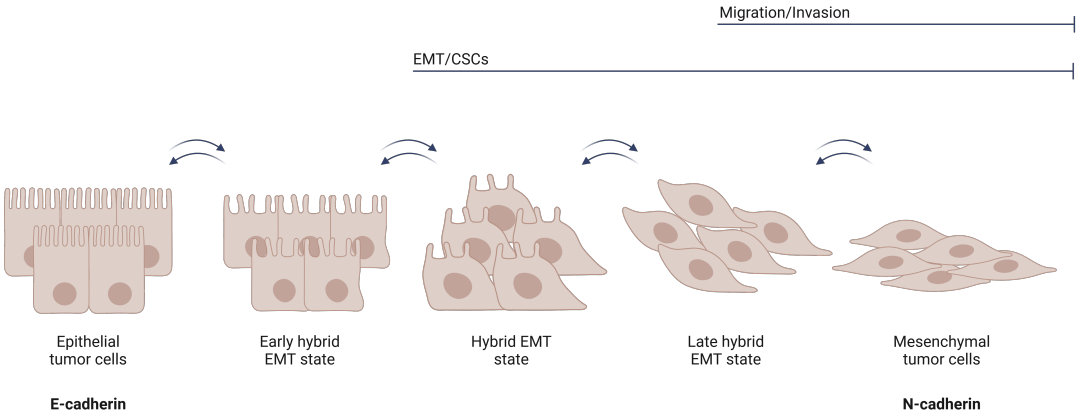

Figure 4. EMT in cancer and its relationship to cadherins expression. The EMT program involves a series of cellular transformations where epithelial cells gradually acquire mesenchymal characteristics due to the activation of a complex network of signaling pathways. This is a dynamic process with multiple intermediate states, that are able to progress and be reverted, according to microenvironment cues. Key molecular changes include the downregulation of epithelial markers, such as E-cadherin, and the upregulation of mesenchymal markers, like N-cadherin. Created with Biorender.

Either partial or complete, EMT activation is affected by inputs from a variety of signaling pathways, which can be grossly divided as inducers of migration and invasiveness, or promoters of stemness. Membrane receptors tyrosine kinase (RTK) are examples of pathways of EMT-induced invasion. Among these, EGFR is central to many cancer types and its activation leads to the activation of the PI3K/Akt, Ras-Raf-MEK-MAPK, JAK/STAT and MEK/ERK signaling pathways [294]. These culminate in the regulation and expression of transcription factors, such as the previously mentioned classical EMT TF, ZEB 1/2, SNAIL 1/2 and TWIST [295,296]. In particular, ZEB1 and ZEB2 repress the epithelial state and activate the mesenchymal state through the regulation of the activity of histone deacetylases, histone

methyltransferases, polycomb and coREST. These show some overlapping effects with SNAIL and TWIST and are also controlled by the activity of these factors [297].

3.1.1. Cadherins in cancer[i]

During oncogenesis in epithelial tissues, organized cell-cell adhesion and normal cell polarity are disrupted by genetic, epigenetic and microenvironmental changes, leading to abnormal signaling, loss of contact inhibition, altered stromal interactions and cell migration (reviewed by [298]). E-cadherin was investigated in seminal studies regarding its role in carcinomas, which are the tumors from epithelial origin. Since then, a variety of studies have proposed CDH1/E-cadherin coding gene as a tumor suppressor gene, by enabling complex mechanisms to promote tissue organization and apoptosis blockade [299–301]. These mechanisms include both biophysical cell-cell adhesion processes, as well as intracellular signaling coupled to inhibition of proto-oncogenic molecules, such as β-catenin and epidermal growth factor receptor (EGFR) [302]. Moreover, suppression of E-cadherin function has been linked to increased cell migration and invasion and induction of a mesenchymal cellular morphology [303]. E-cadherin loss was demonstrated in a variety of cancers, including lobular breast carcinomas [301,302], gastric adenocarcinoma [304], hepatocellular carcinoma [305], melanoma [306], squamous cell carcinomas of the skin [307], as well as esophagus [308] and head and neck carcinomas [309]. More recently, data from other cancers have questioned loss of E-cadherin expression as a ubiquitous marker of disease aggressiveness [310,311]. In later stages, invasive and poorly differentiated breast and ovarian carcinomas, for example, still express E-cadherin, which marks the existence of a mesenchymal to epithelial transition during tumor progression or a hybrid or mixed epithelial-mesenchymal phenotype [312,313].

[i] Noronha C, Ribeiro AS, Taipa R, Castro DS, Reis J, Faria C, Paredes J. Cadherin Expression and EMT: A Focus on Gliomas. Biomedicines 9(10): 1328, 2021

Ever since the initial reports, the role of other cadherins in cancer, including its actions as synergists or antagonists with respect to E-cadherin have been sought. Importantly, the structural diversity of cadherins and, particularly, their tissue-specific expression profiles and actions were indicators that cadherins' role in oncogenesis was most likely heterogeneous. Initial studies in carcinomas have labelled N-cadherin as a "mesenchymal" biomarker; and tied it to the end-stage of a pathological, cancer-related, EMT [263]. Indeed, cadherins frequently exhibit a homotypic binding pattern across cells. For N-cadherin, frequently expressed in stromal and endothelial cells, this leads to epithelial heterotypic cell-cell adhesion, which may facilitate local invasion and dissemination of carcinoma cells [314]. Various studies have elicited the role of N-cadherin in tumor progression [315,316]; however, in others, N-cadherin loss has been linked to a worse prognosis [316].

As for E-cadherin and N-cadherin, P-cadherin cancer-related function seems to be context dependent. In breast cancer, expression of P-cadherin is characteristically observed in tumors with high proliferative rates and decreased cell differentiation, strongly associated with a poor patient survival [317]. By contrast, in colon carcinomas P-cadherin is strongly expressed in well-differentiated, while mostly absent in poorly differentiated colon tumors [318]. P-cadherin overexpression with E-cadherin suppression and N-cadherin induction are considered components of the cadherin switch found in cancer-associated EMT, which is responsible for tumor differentiation and progression. Nevertheless, rising evidence points to the importance of considering the cancer-dependent role of these proteins, as much as the putative interactions between them. In a model of breast cancer, co-expression of P- and E-cadherin was associated with aggressive high-grade breast carcinomas by P-cadherin-mediated disruption of functional cadherin-catenin complexes thereby obstructing the E-cadherin tumor-suppressive role [319].

3.2. EMT-like program in Glioblastoma: the mesenchymal differentiation

A special GBM molecular phenotype with mesenchymal characteristics was first described by Phillips *et al*. 2006. This first appreciation was further solidified with the confirmation of a distinct transcriptomic subtype of GBM, the mesenchymal subtype, with common alterations in *TP53*, *EGFR* and *NF1* genes [87,320]. Since this report, the mesenchymal signature in GBM has been consistently described [321–323], thoroughly studied and frequently correlated with increased aggressiveness, often with multifocal presentation [87,324–327]. An extensive characterization of the mesenchymal gene expression signature identified several transcription factors and their regulators, critical in inducing and sustaining the mesenchymal properties of GBM [321,328]. Notably, the Nuclear Factor-kB (NF-kB) signaling pathway, along with other co-regulating partners, such as STAT3, has been identified as a key driver of the mesenchymal program [328,329]. This pathway directly induces the expression of mesenchymal proteins, including CD44, N-cadherin, and vimentin, while simultaneously promoting an inflammatory microenvironment [193].

The hypothesis of an EMT-like program in GBM remains controversial, although supported by the consistent observation that EMT transcription factors, including ZEB1, are expressed by these tumors [325,330–334]. In addition, several reports have demonstrated that expression of these transcription factors significantly alters GBM dynamics. As an example, the induction of ZEB1 expression through the NF-kB-activated circuit has been shown to disrupt cell-cell adhesion [335], and other reports have similarly reported increases in GBM-cell motility, invasiveness and tumor progression [325,336]. Treatment resistance has also been associated to the expression of EMT-associated TF [322,337,338].

The mesenchymal signature has been proposed to be driven by glioma-intrinsic factors, induced by the tumor microenvironment or by treatment selective pressure [218,321,329,339,340]. For instance, GBM transcriptome has been shown to undergo transitions in response to distinct biological and therapeutic stimuli, intensifying its

heterogeneity and plasticity [329,341]. One well-described transition, referred to as proneural-to-mesenchymal transition, reflects the transcriptomic shift of the proneural subtype into the mesenchymal subtype, frequently associated with therapeutic resistance and accompanied by a concordant phenotypic shift [320,329,342,343]. Indeed, radiation-induced mesenchymal transition in these tumors has been shown to activate NF-kB pathway, expression of C/EBPb and STAT3, upregulation of CD44 and vimentin [322,329,343]. Likewise, temozolomide-associated therapeutic effect appears to contribute to the induction of the mesenchymal transition [141,338,344] and a shift toward the mesenchymal phenotype in tumor recurrence has been concordantly observed [322,338,345]. Failure of anti-angiogenic therapy, which is most commonly used in tumor recurrence, has also been linked to the mesenchymal transformation [341,346,347]. Despite contradicting results suggesting tumor subtype stability upon recurrence, most evidence supports the occurrence of these phenotypic transitions as a common adaptive mechanism of GBM cells in response to treatment exposure and microenvironmental pressure. However, the dynamics underlying the complex relationship between transcription regulation and microenvironmental components remains to be clarified, as does the adding impact of the notorious GBM intratumoral heterogeneity. However, the dynamics underlying the intricate interplay between transcriptional regulation and microenvironmental components remain unclear, particularly regarding the added impact of GBM intratumoral heterogeneity.

Evidence from other tumors suggests that a key property of EMT is the regulation of stem-like features [348]. In malignant gliomas, the existence of a stem-like cancer cell population has been asserted [349], alongside the dynamic regulation of stem cell states within EMT-like activated subpopulations in GBM [330]. As examples, ZEB1 has been shown to promote the expression of neural stem cell markers in GBM while inducing resistance to temozolomide [333]; and Snail expression associated to irradiation resistance drives a stemness phenotype with increase of SOX2 expression [322]. Reciprocally, activation of stem cell signaling pathways leading to invasion and aggressiveness has been shown to partly induce a

mesenchymal transformation in GBM [322,330]. The complex relationship between stemness and EMT is further deepened by the concept of the hybrid epithelial/mesenchymal or intermediate mesenchymal phenotype. In this state, tumor subpopulations exploit both epithelial and mesenchymal traits to enhance tumor aggressiveness and progression (reviewed by [291,350–353]), through a tightly and stepwise regulated process.

3.2.1. Cadherins expression in gliomas[i]

The expression patterns of classical cadherins in gliomas have been evaluated by several, yet contradicting, studies. Gliomas characteristically diffusely infiltrate the underlying cerebral parenchyma, which has normal N-cadherin expression. This behavior reflects a higher tendency to tumor invasion and migration, mechanisms that rely on cell-cell adhesion mechanisms.

N-cadherin expression in gliomas has been described in various patient cohorts, with approximately 60-80% of positive cases in GBM series [354–356]. However, these studies are discordant regarding the correlation between N-cadherin immunoreactivity and patient prognosis. In 1995, Shinoura *et al*. studied the differences in N-cadherin expression in normal brain parenchyma and different gliomas, including pilocytic astrocytomas, low grade oligo or astrocytomas, anaplastic astrocytomas and GBM. Although mRNA levels for N-cadherin were significantly higher in GBM, protein expression was similar among high-grade and low-grade gliomas, as well as in normal brain. Moreover, no consistent association between invasiveness capacity and N-cadherin expression was found [357]. As for Shinoura *et al*., other studies have found discrepant results between N-cadherin mRNA and protein expression levels [358,359]. These results point for the importance of protein stability in the tumor microenvironment. Indeed, a study showed that N-cadherin cleavage by ADAM-10 occurred at

[i] Noronha C, Ribeiro AS, Taipa R, Castro DS, Reis J, Faria C, Paredes J. Cadherin Expression and EMT: A Focus on Gliomas. Biomedicines 9(10): 1328, 2021

a significant higher rate in GBM cells than in normal brain [360], and has been further suggested that N-cadherin cleavage is a pre-requisite for GBM cell migration [360,361].

Other studies have supported a protective role for N-cadherin in gliomas. In glioma models, N-cadherin down-regulation has been associated to altered cell polarization and abnormal motile behavior [362], with a significant increase in tumor cell migration and invasive capacity [362,363]. In surgical specimens, expression levels evaluation pointed to a decrease in N-cadherin immunoreactivity upon GBM recurrence [364,365] and association to tumor CSF dissemination [364]. In line with these results, another report described that cleavage of N-cadherin at the cell membrane by ROBO1, promotes invasiveness of GBM CSC [333].

Nevertheless, contradictory results have questioned the aforesaid role for N-cadherin in gliomagenesis. N-cadherin protein expression levels were also shown to increase according to pathological glioma grade [354,355,366] and to correlate with Ki-67 labelling index [354], suggesting a role for cell adhesion signaling in tumor cell proliferation and dedifferentiation. Importantly, a trend towards decreased survival with increased expression of N-cadherin has also been described in different cohorts [356,366]. In a recent publication, Gritsenko *et al*. studied the mechanisms of brain infiltration by glioma cells, and proposed an AJ-mediated mechanism dependent on N-cadherin and p120-catenin complex, for glioma migration and progression [367]. Similarly, Osuka *et al*. showed an N-cadherin expression increase among the population of radioresistant CSC, with increased stemness and reduced proliferation [368].

E-cadherin expression has been more commonly described as scarce or absent in gliomas [356,366,369,370], while contradicting results regarding its role in disease progression have been hypothesized. Motta *et al*. analyzed E-cadherin mRNA levels in 81 neuroepithelial tumors, including 62 astrocytic tumors, WHO grade I to IV. Low grade astrocytomas (grades I-II) showed higher mRNA levels than did high-grade astrocytomas [371]. Likewise, Bar *et al*. reported E-cadherin staining to be more commonly positive in grade II (43%) over grade IV gliomas (23%) [372]. These results suggest that a decrease in E-cadherin gene expression

underlies astrocytoma progression. Moreover, in a series of low-grade gliomas, *CDH1* promoter methylation status was found to be frequent (65% astrocytomas, 66% oligodendrogliomas and 57% oligoastrocytomas) and hypermethylation status associated with shorter progression-free survival [373]. In the same study, negative immunoreactivity for E-cadherin correlated with shorter patient progression-free and overall survival [373]. Despite these results, the assumption that E-cadherin plays the same protective role in gliomas as in most systemic cancers remains highly dubious. Opposing data documented E-cadherin expression to be higher in high grade gliomas than in low grade gliomas. Furthermore, in specific subtypes of GBM with epithelial and pseudo-epithelial differentiation and high E-cadherin positivity, its expression correlated with a worse prognosis [74,374]. In glioma cell models, E-cadherin expression was confirmed to be a rare event, but to significantly influence in vitro growth and invasion capacity [374].

Taken together, published data point for a broad N-cadherin expression in GBM, with a likely increase from low grade to high grade gliomas [354,355,366]. The tumorigenic result of such expression is nevertheless disputed, as it spans from decreased invasiveness and dissemination capacity [362–364] to worse prognosis with decreased overall survival in clinical series [356]. Data on E-cadherin, whose expression is, on the contrary, fairly absent in gliomas, proposes E-cadherin loss as a potential step in gliomagenesis, as expression is more frequent in low grade gliomas than in GBM [371,372]. Notwithstanding, some reports question such a proposed benign role for E-cadherin in gliomas [374]. It is nevertheless misleading to simply interpret these results in the context of a systemic EMT: they emerge from a collection of small patient series, in which E-cadherin expression is substantially lower and N-cadherin expression considerably higher than in published carcinomas series [317,375–378]. Importantly, no key reciprocal or concomitant down and upregulation for these proteins was explored or suggested; as was not explored the role of cadherin co-expression. Moreover, data on P-cadherin expression in GBM is at the moment inconclusive.

Caution is therefore mandatory when interpreting results for cadherin expression and its role

as EMT biomarkers in GBM, since a proof of a cadherin switching process remains elusive in this pathology and cadherin expression contribution to glioma progression is still unclear **(Figure 5, Table III)**.

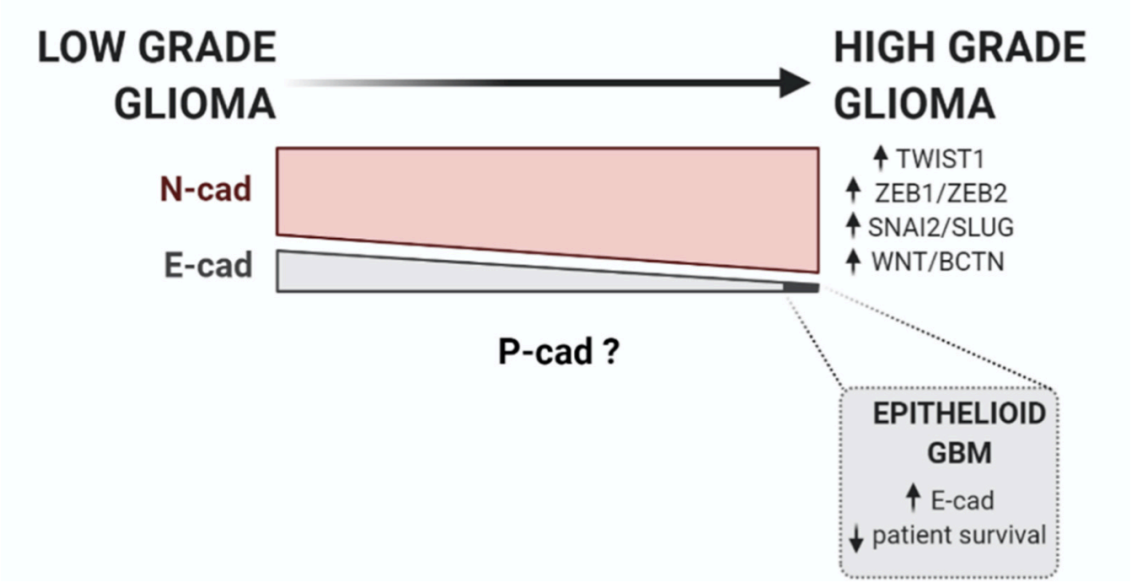

Figure 5. Summary of published literature on cadherins and EMT factors during glioma progression. The progression of low-grade to high-grade glioma is commonly accompanied by an increase in the activity of classical EMT transcription factors and other well-described EMT-promoting pathways, such as ZEB1/ZEB2, TWIST1, WNT/β-catenin pathway, and SNAI2/SLUG. According to current data available, N-cadherin is generally expressed in low-grade glioma, but its expression has been described to be increased in high-grade gliomas. On the contrary, E-cadherin is fairly absent in gliomas. Still, its expression is more frequent in low-grade gliomas than in glioblastoma. Notwithstanding, in aggressive glioblastoma subtypes, with epithelial and pseudo-epithelial differentiation, E-cadherin is found to be overexpressed.[i]

[i] Noronha C, Ribeiro AS, Taipa R, Castro DS, Reis J, Faria C, Paredes J. Cadherin Expression and EMT: A Focus on Gliomas. Biomedicines 9(10): 1328, 2021

Table III. Summary of cadherin expression and prognosis in different published glioma series.

Author/year	Cadherin and method	Result	Prognosis
Shinoura *et al* 1995	N-cadherin. mRNA and protein level in 21 gliomas (one pilocytic astrocytoma, 4 grade II astrocytomas, 3 grade III anaplastic astrocytoma, 9 GBM, 2 mixed gliomas, 2 grade II oligodendroglioma)	Ncad expression equal (protein) to higher (mRNA) in GBM	No prognosis
Asano *et al* 1997	N- and E-cadherin. Immunohistochemistry of 22 astrocytomas (13 GBM and 9 anaplastic grade III astrocytoma)	No Ecad expression Ncad expression in all primary tumors. Ncad loss associated to tumor dissemination.	Ncad staining better prognosis.
Schwechheimer *et al* 1998	E-cadherin. Immunohistochemistry of 42 gliomas and other 105 SNC tumors	No Ecad staining in gliomas	-
Howng *et al* 2002	E-cadherin. expression by RT-PCR in 16 astrocytomas and 29 other SNC tumors	Ecad staining in 2/16	No prognosis
Utsuki *et al* 2002	E- and N-cadherin. Immunohistochemistry of 45 gliomas (18 GBM, 16 grade III anaplastic astrocytomas, 11 grade II diffuse gliomas).	No Ecad staining Ncad staining in GBM (81%) and anaplastic astrocytoma (31%) - Ncad staining increases with WHO grade.	Ncad staining associated to a worse prognosis
Motta *et al* 2008	E-cadherin. RT-PCR in 62 astrocytomas (21 grade I astrocytomas, 10 grade II astrocytomas, 10 grade III astrocytomas and 21 GBM) and 19 other SNC tumors	Ecad expression higher in low grade astrocytomas than high grade astrocytomas. Ecad expression decreases with WHO grade.	Ecad expression associated with a better prognosis
Rodriguez *et al* 2007	E-cadherin. Immunohistochemistry of 58 GBM with adenoid, epithelioid, or true epithelial features	Ecad staining in 82% of GBM.	No prognosis
Lewis-Tuffin *et al* 2010	E-cadherin. Immunohistochemistry of TMAs of 83 cases of GBM or anaplastic astrocytoma and 31 gliomas and immunohistochemistry of 27 GBM with epithelial/pseudo-epithelial differentiation	No Ecad expression in TMA Ecad staining in 33% of GBM with epithelial/pseudo-epithelial differentiation. Worse overall survival in patients with Ecad expression.	Ecad staining associated with a worse prognosis.
D'Urso *et al* 2011	E-cadherin. *Ecad* gene (CDH1) promoter methylation and E-cad expression by methylation-specific polymerase chain reaction (MSP) and immunohistochemistry in 84 low-grade gliomas (43 diffuse astrocytomas, 27 oligodendrogliomas and 14 oligoastrocytomas)	*CDH1* promoter hypermethylation correlated with a worse prognosis. Ecad expression positive in 15/43 astrocytomas, 9/27 oligodendrogliomas and 6 /14 oligoastrocytomas. Loss of immunoreactivity for E-cadherin correlated with worse survival	Loss of Ecad expression worse prognosis
Wu *et al* 2013	E- and N-cadherin. Immunohistochemistry of 40 brainstem gliomas (30 low grade and 10 high-grade) and RT-PCR of 18 brainstem gliomas (10 low grade and 8 high grade)	No Ecad staining, weak mRNA levels. N-cad expression increased with WHO grade and showed a trend toward shorter survival.	Ncad expression associated to a worse prognosis

Table III. Summary of cadherin expression and prognosis in different published glioma series (cont.)

Author/year	Cadherin and method	Result	Prognosis
Bar *et al* 2014	E-cadherin. immunohistochemistry of 92 gliomas (23 grade I pilocytic astrocytoma, 23 grade II astrocytoma, 7 grade II oligodendroglioma, and 39 GBM.	Ecad staining in 28.8% of gliomas, no differences with WHO grade.	No prognosis
Darweesh *et al* 2016	N-cadherin. Immunohistochemistry of 60 GBM	Ncad expression in 88.3% cases. Ncad expression associated with tumor histological variant - more common in cases of Gliosarcoma than in glioblastoma with oligodendroglioma component.	No prognosis
Noh *et al* 2017	E- and N-cadherin Immunohistochemistry of 92 gliomas	Ecad expression in 8.7% Ncad expression in 88.0% No significant difference in OS, PFS increased in low Ncad expression.	Tendency for worse prognosis with Ncad expression

3.3. EMT and the Immune System

Cancer immunosurveillance refers to the recognition and destruction of cancer cells by the innate and adaptive immune system. Evidence increasingly indicates that tumor cells' ability to evade immunosurveillance reflects mechanisms tightly regulated by the tumor microenvironment, including its metabolic reprogramming and the cellular heterogeneity/plasticity inherent to tumors. The EMT program is therefore easily considered as a key modulator of this advantageous phenotypic variability.

Data from various types of cancer suggest a link between EMT and immunosuppression within the tumor microenvironment. For instance, EMT activation in non-small cell lung cancer (NSCLC) associates with an increase in Treg population and expression of ICP molecules, with a consequent increase in local immunosuppression [379]. Similarly, the mesenchymal subtype of ovarian cancer exhibits fewer tumor-infiltrating T cells [380]. Consistent evidence supports that cells undergoing EMT are able to regulate the tumor immune microenvironment through the production of cytokines and recruitment of immunosuppressive cells [381,382], while, in turn, immune regulation within the tumor microenvironment induces EMT in cancer cells [382]. The bidirectional crosstalk strongly amplifies both mechanisms, culminating in an increase in tumor aggressiveness, dissemination capacity and treatment resistance [383].

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CHAPTER II. Hypothesis and Aims

Hypothesis and Aims

Glioblastoma (GBM) is a rare incurable cancer, for which there is an urgent need of effective novel therapies. In the last years, a multitude of recent phase III trials did not meet their pre-specified primary end points. This is at least partly explained by the direct adaptation of successful treatments in systemic cancer to a disease that has a unique biology and a particular microenvironment. For instance, GBM is a tumor with low immunogenicity but, at the same time, with a striking immunosuppression. These characteristics impose particular challenges and strategies for the success of immunotherapy. In addition, as a notoriously heterogeneous disease, the identification of biomarkers capable to predict tumor subtypes and therapy response is of utmost importance in GBM.

The main goal of our work was to explore the common ground between mechanisms of tumor evasion and the activation of epithelial-to-mesenchymal transition (EMT) programs in GBM, with a key interest in identifying biomarkers that reflect the interplay between these two mechanisms.

In order to answer this question, we established the following specific aims:

- To establish a large cohort of GBM patients with detailed demographic, clinical, neuropathological and treatment characteristics. Extensive documentation of initial MRI features aims to identify preoperative biomarkers, while evaluation of survival statistics intends to identify predictors of prognosis.

- To characterize PD-L1 expression in a series of GBM, evaluate its correlation to patient prognosis and identify clinical predictors of PD-L1 expression. In addition, we aim to study associated systemic inflammation and immunosuppression.

- To define an EMT-like program in GBM. EMT is characterized by classical cadherins' alterations that are not well defined in gliomagenesis. We therefore aim to characterize cadherins' expression in GBM and to evaluate whether they are useful as markers of different EMT stages for this tumor. In addition, we aim to analyze ZEB1/ZEB2 expression in our series, hypothesizing that the combined expression of cadherins and EMT-transcription factors will reflect a specific EMT program in GBM.

- To explore the crosstalk between the EMT and the immune system in GBM. Specifically, our goal is to analyze whether the PD-L1-associated immunosuppression and inflammatory microenvironment associates with distinct EMT stages.

CHAPTER III. Clinical Predictors in a series of 313 glioblastoma patients: Insights and Implications[i]

[i] Noronha C, Pinto C, Fernandes A, Magalhães M, Simões J, Ramos C, Pinheiro C, Silva E, Calheiros A, Taipa R, Reis J. Clinical Predictors in a series of 313 glioblastoma patients: Insights and Implications, 2025. *Submitted for publication*

Clinical Predictors in a series of 313 glioblastoma patients: Insights and Implications

1. Introduction

Glioblastoma (GBM), the most prevalent malignant primary brain tumor in the adult, presents significant challenges in oncological care. The annual incidence is approximately 3,2 cases per 100.000, and the median age at diagnosis is 65(1). Despite recent advances in treatment modalities, the prognosis for GBM patients remains poor, denoted by a median overall survival (OS) of approximately 11 months and a 5-year survival rate below 6% (2).

The clinical presentation of patients with GBM is often non-specific and progressive in nature, although sometimes mirrors the functional aspect of the affected brain area as focal neurological deficit. Headache is a frequent and early symptom, and its characteristics vary according to tumor size and growth rate. Seizures occur as an initial symptom in approximately 20% of patients with GBM, with an additional 20% experiencing seizure onset at a later stage in the disease progression (3,4). Other symptoms may include neuropsychological symptoms and mental status alteration. Clinical presentation has been suggested to associate with patient survival (5–7).

In addition to clinical presentation, recent studies have explored the relationship between pre-operative MRI characteristics and survival outcomes in GBM patients using qualitative and quantitative characteristics, as well as radiomics (8,9). Visually Accessible REMBRANDT Images (VASARI) metrics is a validated approach, and several of its features, such as tumor location, deep white matter invasion and ependymal extension, proportion of necrosis or non-enhancing component, have been correlated to patient prognosis and thus pointed as preoperative factors of prognosis (10–13).

Consistently identified prognostic factors linked to poor survival in GBM include advancing

age, poor performance status (PS) and limited extent of surgical tumor resection (14–16). Determining how best to use these prognostic characteristics, alongside other clinical and imaging features, to guide treatment decisions remains indefinite. In particular, treatment of the elderly patients poses specific challenges, as they often present higher rates of medical comorbidities, reduced tolerance to treatment toxicities, and, unequivocally, present an overall worse prognosis (17,18).

Another important issue in GBM is the early deterioration in the postoperative phase observed in some patients. It has been more commonly associated with negative effects from surgery and no clinical predictors have been proposed to date (19–21).

The combination of a devastating disease with the potential for serious surgical hazards, with a severe deleterious effect on patient autonomy and quality of life, urges for the identification of characteristics which can improve preoperative prognosis stratification. In this retrospective study, we meticulously analyze patient demographics, clinical and MRI characteristics, surgical approaches, and outcomes, aiming to elucidate prognostic information among established risk factors. Additionally, we explored subgroups less frequently delineated in existing literature, thereby addressing the complexity encountered in treatment decisions. Our study thus aims to provide insights into the multifaceted aspects of glioblastoma patients.

2. Methods

2.1. Patient selection, demographic and clinical characteristics

A surgical cohort of patients diagnosed with GBM in Hospital de Santo António - Unidade Local de Saúde de Santo António, from January 2010 until December 2017 was reviewed. Exclusion criteria included age inferior to 18 years-old and infratentorial tumors. All selected tumors were reviewed and those not compatible with GBM or sparse GBM specimens mostly

composed of necrosis or tumor-invaded parenchyma were also excluded **(Figure 1)**. A total of 313 patients were included. Of these, 135 (43,13%) had available IDH mutation data and a positive mutation rate of 3 %. Date of last follow-up was August of 2021.

Clinical records were reviewed in order to document patient demographics, presenting symptoms and functional status upon presentation, after surgery and after 12 months. Preoperative MRI were reviewed and qualitative and quantitative characteristics according to Visually Accessible Rembrandt Images (VASARI) features were documented and validated by an experienced neuroradiologist. Treatment decision (surgery and adjuvant treatments) and patient follow-up was documented with detail, as well as progression-free (PFS) and overall survival (OS) times. This study has been performed in accordance with the international Helsinki declaration. Moreover, it has also been approved by the ethics committee at Unidade Local de Saúde de Santo António (DEFI 104-17 (093-DEFI/091-CES).

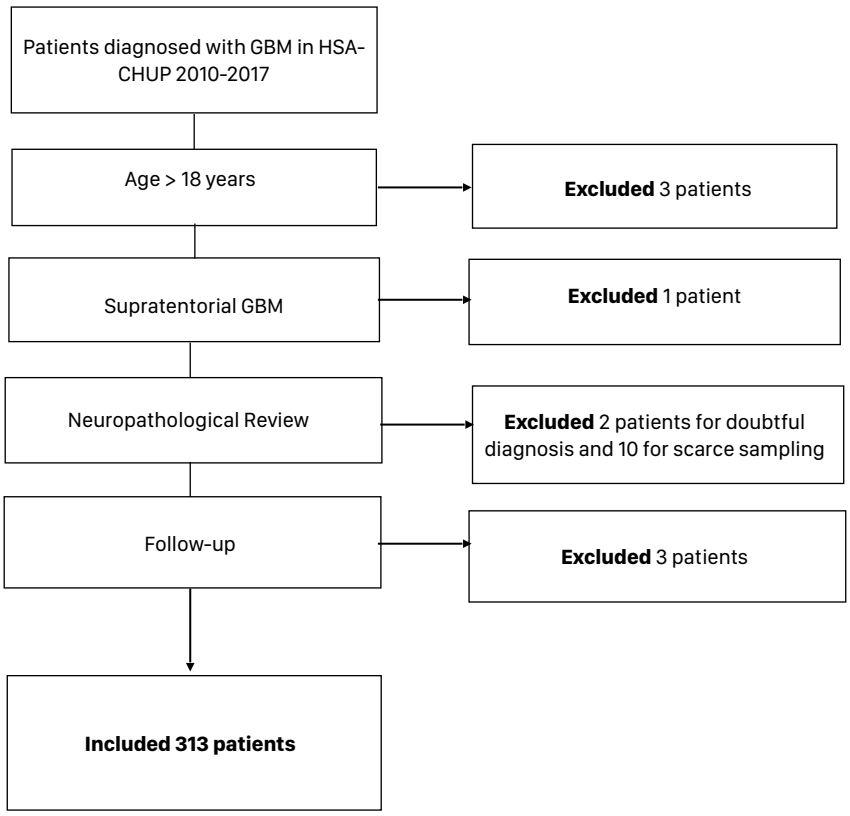

Figure 1. Patient selection criteria.

2.2. Treatment decision

The decision to pursue surgical resection versus needle biopsy was determined either by a consensus between various neurosurgeons or by a multidisciplinary team for patients with worse functional status, and was based in patient traits, autonomy and MRI characteristics **(Supplementary Table I)**. Use of adjuvant treatments was based on the recommendations of a multidisciplinary team dedicated to neuro-oncology. Post-operative MRI was available for 54 patients and extent of resection was calculated. Resection was codified as: gross-total (GTR) when over 98% of enhancing-tumor was removed, subtotal resection (STR) in over 80% and partial resection (PR) when extent of resection was inferior to 80%(22).

2.3. Statistics

The chi-square test was used to assess group differences, as appropriate. Survival as a function after surgical resection or biopsy was expressed as estimated Kaplan-Meier plots and differences assessed via log-rank analysis. The univariate Cox proportional hazards model was used to determine hazard ratios of each variable as a predictor of OS and PFS. Variables with a p-value <0.05 in the univariable analysis was selected for multivariable Cox regression analysis. A 2-tailed significance level (p-value) of 0.05 was applied.

3. Results

3.1. Patient population and treatment characteristics

Our series comprehends a total of 313 patients with a mean age at diagnosis of 61,743 ± 10,483 years old. We did not observe a trend towards mean age alteration at diagnosis throughout our study timeframe. The youngest patient was 19 years old and the oldest 85 years old. The majority of patients were male (57%, n=178) **(Table I).**

Table I. Demographics and clinical characteristics of the patients with GBM.

		Mean, range	n	%
Age		61,743 ± 1,483 (19 -83)		
Karnofsky Performance Status		74 (50-100)		
Lymphocyte count		1722 ± 192,78		
NLR		9.654 ± 0.917		
Gender	**F**		135	43.1
	M		178	56.9
Corticoids before resection	**Yes**		140	44.7
	No		173	55.3
Extent of surgical resection	**Gross Total**		95	30.4
	Subtotal		82	26.2
	Partial		16	5.1
	Biopsy		120	38.3
1st line treatment	**No**		87	27.8
	Radiotherapy		15	4.8
	RT + Temozolomide		8	2.5
	Stupp protocol		203	64.9
Resection at recurrence	**Yes**		39	12.5
	No		274	87.5
Progression Free Survival		4 months (0-96)		
Overall Survival (OS)		9 months (1-124)		
OS with surgery + Stupp protocol		21 months (10-124)		

Functional status at diagnosis was defined using Karnofsky Performance Status (KPS). At presentation, median (IQR) KPS was 80 (70-90). The gross majority (88%) of patients in our

series was considered independent at diagnosis (KPS 70-100). Surgical removal of the lesion was considered in patients with functional dependency when the autonomy loss was attributed to the mass effect from the lesion or associated perilesional edema (n=38, 12,1%).

Focal neurological deficits was the most common presentation, occurring in 110 patients (35,6%), followed by behaviour alterations in 66 patients (21,4%) and headache in 65 patients (21%). Over 17% of patients presented with seizures and in 4 patients the tumor was diagnosed incidentally during imaging exams performed due to trauma or unrelated conditions **(Table I).**

Average major axis was 6,18 ± 2,09 cm and minor axis 3,8 ± 1,9 cm. The majority of tumors were located in the frontal (n=112, 36,1%) and temporal (n=95, 30,4%) lobes. Seventeen tumors (4,8%) were not located in cerebral hemispheres and were codified as deep lesions, considering their thalamic or basal ganglia location. Up to a fifth of tumors (n=71, 22,7%) were multilobar at presentation **(Table I)**. We identified 76 tumors as multifocal tumors (24,3%), 89 tumors had ependymal extension (28,4%), 92 tumors had corpus callosum extension (29,4%), including 38 butterfly GBM (12,1%). Cysts were present in 49 tumors (15,7%) **(Table I).**

Brain tumor biopsy alone was performed in 136 patients (43.5%) and surgical resection in 177 patients (56.5%) **(Table I, Supplementary Figure 1).** Characteristics associated with biopsy decision are detailed in **Supplementary Table I.** Total resection was achieved in 44,4%, subtotal resection in 40,7% and partial resection in 14,8% of patients **(Supplementary Figure 1D).** Complications were observed in a total of 19 patients (6%).

Postoperative adjuvant treatment relied in the standard of care, chemotherapy (temozolomide) and radiotherapy (RT), according to Stupp protocol, for the majority of the patients (189 patients, 60,4%). Other less frequent treatment options included sequential radiotherapy followed by chemotherapy with temozolomide in 8 patients (2,5%) and radiation alone in 19 patients (6,1%) **(Table I).**

On the date of the last follow-up, 297 patients had recurred (94,9%). Repeated resection upon first recurrence was performed in 38 patients (12,8%) and in 6 patients (2%) upon second

recurrence. Second-line treatments were initiated in 159 patients (53,5%) **(Table I).**

3.2. Long term outcomes

At last follow-up, 297 patients had recurred and 296 patients had died. Median PFS was 4 months and median OS in our series was 10 months. Surgical resection significantly improved PFS and OS to 6 months and 14 months, respectively **(Supplementary Figure 1B-1C)**. Likewise, extent of resection significantly stratified patient survival times, with median OS of 22 months for patients with total resection **(Supplementary Figure 1E-1F).** Patients who underwent surgical resection and adjuvant treatment with Stupp protocol (n= 138, 44%) had 7 months and 16 months of PFS and OS, respectively. We identified 21 patients (6,7%) with a long-term survival (greater than 36 months) in our series.

3.3. Patient characteristics correlated with prognosis

Univariate analysis of baseline clinical features identified 3 characteristics associated with patient outcome: age, initial performance score and clinical presentation **(Table II)**.

Table II. Univariate analysis of initial clinical characteristics.

		Progression Free Survival					Overall Survival				
		Median (months)	HR	95,0% CI		p-value	Median (months)	HR	95,0% CI		p-value
				Inferior	Superior				Inferior	Superior	
Age			1,016	1,004	1,029	**0.009**		1,023	1,012	1,035	**<0.001**
Gender	**Female**	8	1				12	1			
	Male	5	0,813	0,63	1,051	0.114	8	1,042	0,827	1,313	0.725
KPS	**90-100**	5	1				12	1			
	80	5	1,091	0,812	1,467	0.563	10	1,024	0,782	1,3410	0.863
	70	4	1,49	1,019	2,180	**0.040**	5	1,603	1,131	2,272	**0.008**
	<70	3	1,441	0,935	2,221	0.098	5	1,794	1,226	2,625	**0.003**
Presentation	**Seizure**	8	1				12	1			
	Focal	3	1,620	1,104	2,378	**0.014**	7	1,592	1,130	2,244	**0.008**
	Gait	2	3,345	1,755	6,378	**<0.001**	5	2,926	1,602	5,344	**<0.001**
	Headache	5	1,502	0,990	2,281	0.056	11	1,389	0,951	2,028	0.089
	Behaviour	5	1,577	1,029	2,416	**0.037**	8	1,487	1,020	2,167	**0.039**

Patient presentation was dictated by various tumor characteristics on initial MRI. For instance, patients with larger tumors more frequently presented with behaviour alterations (p=0.014), while seizures was the most common presentation for the smaller ones (p<0.001). Among the distinct clinical presentations, gait imbalance and seizures appear as indicators of prognosis **(Figure 2A and 2B, Table III)**. In particular, patients who presented with gait imbalance experienced the shortest times for recurrence (p<0.001, median 2 months) and worse OS (median 5 months, p=0.004). On the other hand, presentation with seizures associated with significantly longer PFS (median 8 months, p=0.003) and OS (median 12 months, p=0.006) **(Figure 2A and 2B, Table III).**

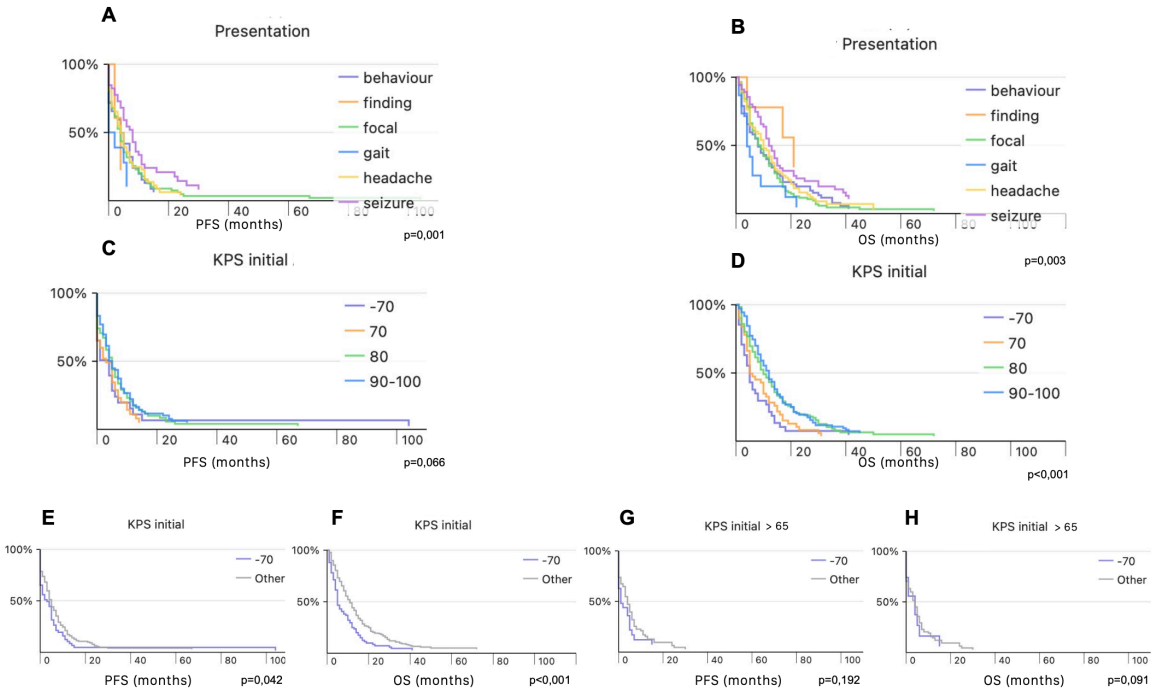

Figure 2. Clinical presentation and initial patient autonomy. Clinical presentation **(2A and 2B)** and initial autonomy (KPS) **(2C-2F)** are predictors of time to recurrence and overall survival in our cohort. In particular, preserved self care appears to be a cut-off able to predict longer times to recurrence **(2E)** and overall survival **(2F)**. Importantly, KPS was not helpful in prognosis prediction in the elderly population (>65 years old) **(2G and 2H)**.

Table III. Imaging and treatment characteristics across clinical presentation subgroups.

	GBM series	Behaviour		Focal deficit		Seizures		Headache		Gait	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value
	313 (100%)	66 (21,4%)		110 (35,6%)		54 (17,5%)		65 (21%)		14 (4,5%)	
MRI features											
Major axis (mean in cm)	6,18 ± 2,09	7,01 ± 3,43	**0.014**	6,53 ± 3,15	0.249	4,38 ± 2,46	**<0.001**	6,37 ± 3,36	0.753	5,93 ± 3,19	0.782
Location		Frontal 40%	0.230	Frontal 37% Parietal 27%	0.092	Frontal 48%	0.091	Temporal 44,6%	**0.014**	Deep 21%	0.060
Eloquent	124 (39,6%)	19 (28,8%)	**0.034**	59 (53,6%)	**<0.001**	26 (48,1%)	0.186	15 (23,1%)	**0.002**	5 (35,7%)	0.730
Multifocal	76 (24,3%)	16 (24,2%)	0,995	26 (23,6%)	0,846	10 (18,5%)	0,278	16 (24,6%)	0,942	7 (50%)	**0,022**
Ependymal extension	89 (28,4%)	25 (37,9%)	0,056	31 (28,2%)	0,931	7 (13%)	**0,005**	22 (33,8%)	0,281	3 (21,4%)	0,550
Cortical involvement	202 (64,5%)	40 (60,6%)	0,468	65 (59,1%)	0,147	45 (83,3%)	**0,001**	41 (63,1%)	0,802	8 (57,1%)	0,562
CET crosses midline	38 (12,1%)	15 (22,7%)	**0.004**	9 (8,2%)	0.101	6 (11,1%)	0.770	6 (9,2%)	0.397	2 (14,3%)	0.817
Surgery	177 (56,5%)	37 (56,1%)	0.836	58 (52,7%)	0.242	33 (61,1%)	0.523	42 (64,6%)	0.175	7 (50%)	0.580
Adjuvant treatment	216 (69%)	41 (62,1%)	0.174	73 (66,4%)	0.458	43 (79,6%)	0.064	50 (76,9%)	0.122	5 (35,7%)	**0.006**
median PFS (months)	4	4	0.640	3	0.295	8	**0.003**	4	0.938	2	**<0.001**
median OS (months)	10	8	0.601	8	0.114	12	**0.006**	10	0.896	5	**0.004**

Initial autonomy and functional status were also significantly associated to clinical presentation ($p<0.001$). For instance, patients with poor functional status (KPS <70) were comparatively more likely to present with behaviour alterations and gait imbalance **(Table IV).** Imaging features correlated with worse autonomy status upon diagnosis included deep-seated lesions, multifocality and bilateral contrast-enhancing lesions **(Table IV)**. Similarly to clinical presentation, initial KPS stratified patient overall survival **(Figure 2C and 2D, Table IV)** and preserved self-care appears as a key factor for outcome prediction in our cohort (PFS, $p=0.042$, and OS, $p<0.001$) **(Figure 2E and 2F)**. However, it is important to note that initial KPS was a less reliable predictor of outcome in the elderly population **(Figure 2G and 2H)**.

Table IV. Clinical and treatment characteristics with distinct initial functional status.

	GBM series	KPS <70	KPS ≥ 70	
	n (%)	n (%)	n (%)	p-value
	313 (100%)	38 (12%)	275 (88%)	
Presentation				
Behaviour	66 (21,4%)	15 (39,5%)	51 (18,8%)	**0.004**
Focal deficit	110 (35,6%)	10 (26,3%)	100 (36,9%)	0.202
Seizures	54 (17,3%)	2 (5,3%)	52 (19,2%)	**0.034**
Gait	14 (4,5%)	4 (10,5%)	10 (3,7%)	0.058
Headache	65 (21%)	7 (18,4%)	58 (21,4%)	0.673
MRI features				
Major axis (mean in cm)	6,18	6,48 ± 3,55	6, 14 ± 3,23	0.365
Eloquent	124 (39,6%)	15 (39,5%)	109 (39,6%)	0.985
Deep lesions	15 (4,8%)	6 (15,8%)	9 (3,3%)	**<0.001**
Multifocal	76 (24,3%)	17 (37,8%)	59 (22%)	**0.022**
Ependymal extension	89 (28,4%)	15. (39,5%)	74 (26,9%)	0.108
Cysts	49 (15,7%)	8 (21,1%)	33 (12%)	0.121
CET crosses midline	38 (12,1%)	10 (22,2%)	28 (10,4%)	**0.025**
Surgery	177 (56,5%)	13 (34,2%)	166 (60,4%)	**0.002**
Adjuvant treatment	216 (69%)	18 (47,4%)	198 (72%)	**0.002**
Recurrence during treatment	141 (64,7%)	13 (72,2%)	128 (64%)	0.485
median PFS (months)	4	2	4	0.276
median OS (months)	9	5	10	**0.005**

Finally, age significantly associated with PFS and OS in our univariate analysis and Kaplan-Meier plots. Our series included a total of 140 (44,7%) elderly patients, as defined by age >65 years old. In order to explore an age-relevant cut-off, we considered 4 different older age subgroups: >65 years-old (n=140), >70 years old (n=83), >75 years old (n=35) and >80 years old (n=7) **(Table V)**.

Table V. Comparison of clinical characteristics and survival statistics across different age groups.

	GBM series	>65		>70		>75		>80	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value
	313 (100%)	140 (44,7%)		83 (26,5%)		35 (11,2%)		7 (2,24%)	
Presentation									
Behaviour	65 (20,8%)	33 (23,6%)	0,271	23 (28,7%)	**0,041**	9 (26,5%)	0,385	1 (14,3%)	0,669
Focal deficit	116 (37,1%)	65 (46,4%)	**0,002**	31 (38,8%)	0,717	16 (47,1%)	0,201	3 (42,9%)	0,748
Seizures	54 (17,3%)	20 (14,3%)	0,211	15 (18,8%)	0,681	6 (17,6%)	0,949	3 (42,9%)	0,07
Headache	74 (23,6%)	21 (15%)	**0,001**	11 (13,8%)	**0,016**	3 (8,8%)	**0,031**	0	0,137
KPS preop <70	38 (12,1%)	16 (11,4%)	0.729	11 (13,8%)	0.609	6 (17,6%)	0.298	0	0.320
MRI features									
Deep lesions	15 (4,8%)	4 (2,9%)	0,149	2 (2,5%)	0,266	2 (5,9%)	0,753	0	0,548
Multifocal	76 (24,3%)	36 (25,7%)	0,595	21 (26,2%)	0,634	6 (17,6%)	0,339	3 (42,9%)	0,246
CET crosses midline	38 (12,1%)	14 (10%)	0.297	11 (13,8%)	0.609	5 (14,7%)	0.628	0	0.320
Ependymal extension	89 (28,4%)	42 (30%)	0,581	23 (28,7%)	0,942	9 (26,5%)	0,788	2 (28,6%)	0,994
Surgery	177 (56,5%)	72 (51,4%)	0,064	38 (47,5%)	**0,042**	15 (44,1%)	0,103	2 (28,6%)	0,122
KPS posop <70	49 (15,7%)	23 (16,4%)	0,735	17 (21,2%)	0,110	9 (26,5%)	0,066	2 (28,6%)	0,342
Reduced KPS posop	71 (22,7%)	40 (28,6%)	**0,025**	23 (28,7%)	0,133	10 (29,4%)	0,321	3 (42,9%)	0,197
Adjuvant treatment	216 (69%)	92 (65,7%)	0,257	44 (55%)	**0,001**	15 (44,1%)	**<0,001**	2 (28,6%)	**0,022**
Adjuvant treatment after surgery	148 (82,7%)	57 (79,2%)	0,308	28 (73,7%)	0,099	9 (60%)	**0.015**	1 (50%)	0.219
Recurrence during treatment	141 (64,7%)	69 (75%)	**0,006**	32 (72,7%)	0,211	13 (86,7%)	0,065	2 (100%)	0,294
median PFS (months)	4	4	0.053	3	**0.037**	2	0.123	1	**0.027**
median OS (months)	10	8	**0.003**	6	**0.026**	5	**<0,001**	4	**<0,001**
median PFS with surgery + QTRT (months)	7	6	0.159	5	0.069	6	0.618	-	
median OS with surgery + QTRT protocol (months)	16	14	0.069	16	0.685	12	0.179	-	

Minor differences were identified in clinical presentation, as focal deficits were significantly more common as a presenting symptom in older age patients, and epilepsy was less frequent. Despite this, we did not observe differences in initial functional status or initial MRI features

(Table V). With increasing age, patients were gradually less frequently considered for surgical resection **(Table V).** In the postoperative period, we documented higher rates of autonomy decline in the elderly population, which occurs in over 28% of patients aged >65 (p=0.025) and almost a half of patients aged >80. Adjuvant treatment was also significantly less likely to be initiated in these age groups, particularly in patients >75 and >80 years old. Moreover, we noted this tendency also in the elderly patients submitted to surgery **(Table V)**. Tumor recurrence rates during adjuvant treatment were significantly higher for patients >65 years old. Survival analysis identified significantly shorter times for recurrence for patients >70 (p=0.037) and >80 (p=0.027) years old, and a tendency for patients aged >65 (p=0.053). Overall survival was significantly reduced for all age groups (>65, p=0.003; >70 p=0.026; >75, p<0.001 and >80, p<0.001) **(Table V, Figure 3)**.

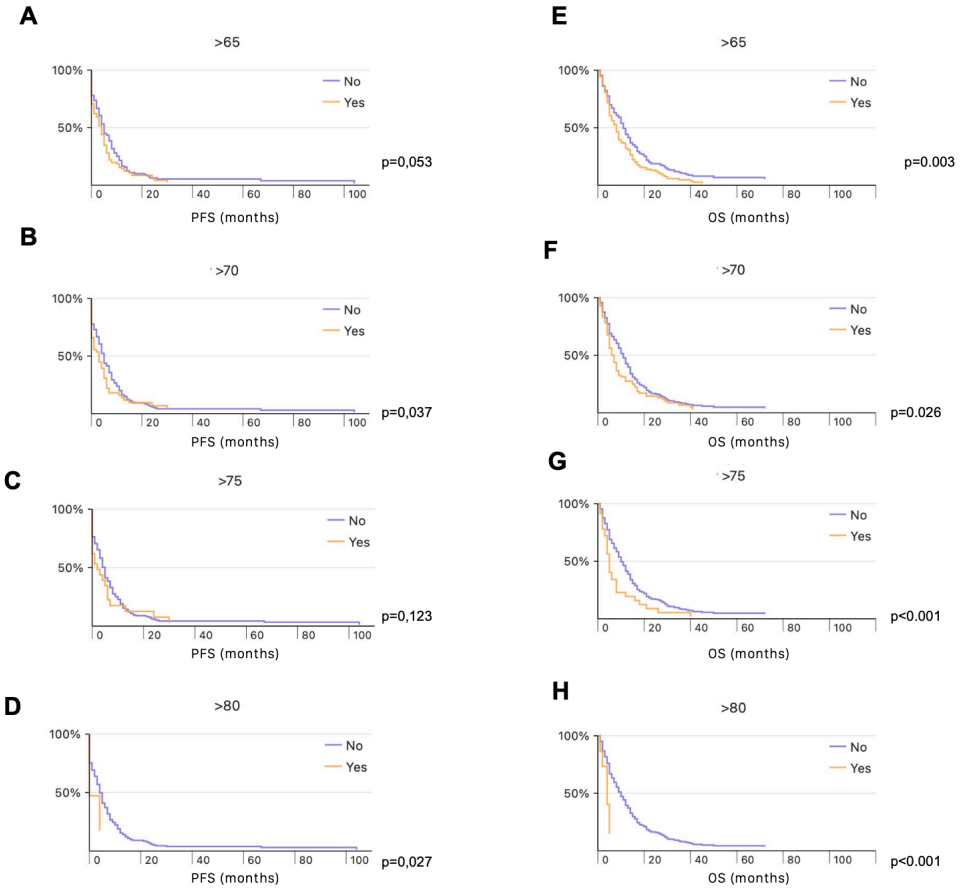

Figure 3. Survival analysis in the elderly population. Evaluation of PFS **(3A-D)** identifies significant shorter recurrent times for patients older than 70 and those older than 80 years old. Overall survival **(3E-H)** consistently identifies worse survival times for the elderly population, in all different cut-off.

Finally, 138 patients completed the best standard of care, surgery followed by adjuvant chemotherapy + radiotherapy. Of these, 49 were >65 years old, 22 were >70 years old and 5 were >75 years old. Among patients who underwent surgery followed by adjuvant treatments, no significant differences in survival were observed, albeit a tendency was observed in PFS for patients >70 years (p=0.069) and in OS for patients >65 years (p=0.069) **(Table V)**.

3.4. MRI features predictive of prognosis

In the univariate analysis of the VASARI qualitative and quantitative imaging features **(Table VI)**, we found multiple imaging features that were significantly associated with both OS and PFS. The presence of cysts was the only characteristic identified with an improved prognosis (PFS, p=0.038 and OS, p=0.028).

TABLE VI. Univariate analysis of MRI features.

		Progression Free Survival					Overall Survival				
		Median (months)	HR	95,0% CI		p-value	Median (months)	HR	95,0% CI		p-value
				Inferior	Superior				Inferior	Superior	
Major axis (mm)			1					1			
			0,999	0,992	1,005	0.756		1,003	0,998	1,007	0.203
Minor axis (mm)		5	1				11	1			
		4	0,991	0,978	1,003	0.141	8	1,002	0,995	1,009	0.607
Location	**Temporal/ Frontal/ Parietal**	5	1				12	1			
	other	3	0,845	0,56	1,274	0.421		1,161	0,816	1,653	0.407
Laterality	**Left**	8	1				12	1			
	Right	5	1,008	0,783	1,297	0.951	8	0,983	0,781	1,236	0.880
Eloquent	**No**	5	1				12	1			
	Yes	1	0,942	0,729	1,212	0.647	5	0,997	0,791	1,257	0.983
Enhancement quality	**No/Mild**	5	1				11	1			
	Marked	3	1,079	0,691	1,685	0.739	7	1,263	0,847	1,882	0.251
Cyst	**No**	4	1				9	1			
	Yes	7	0,674	0,442	1,026	**0.038**	14	0,666	0,444	0,998	**0,028**
Multifocal	**No**	5	1				12	1			
	Yes	3	1,877	1,397	2,521	**<0.001**	5	2,293	1,753	2,999	**<0.001**

TABLE VI. Univariate analysis of MRI features (cont.).

		Progression Free Survival					Overall Survival				
		Median (months)	HR	95,0% CI		p-value	Median (months)	HR	95,0% CI		p-value
				Inferior	Superior				Inferior	Superior	
T1/FLAIR ratio	**Infiltr./Mixed**	6	1				14	1			
	Expansive	2	1,159	0,831	1,615	0.385	5	1,289	0,958	1,734	0.094
Thickness of enhancing margin	**Thin**	6	1				14	1			
	Thick/ Nodular/ Solid	0	1,869	0,775	4,508	0.164	4	1,881	1,045	3,387	**0.035**
Definition of the enhancing margin	**Poorly**	6	1				14	1			
	Well	0	0,658	0,323	1,34	0.248	4	0,966	0,607	1,538	0.884
Definition of the non-enhancing margin	**Poorly**	6	1				14	1			
	Well	0	0,722	0,372	1,402	0.336	4	1,124	0,711	1,775	0.617
Hemorrhage	**No**	5	1				11	1			
	Yes	4	0,87	0,621	1,217	0.415	8	0,982	0,712	1,355	0.914
Proportion of necrosis	**<33%**	5	1				12	1			
	>33%	2	0,774	0,599	0,889	0.052	5	0,887	0,705	1,115	0.304
Proportion of edema	**<33%**	5	1				22	1			
	>33%	3	1,137	0,587	2,204	0.703	5	1,008	0,609	1,667	0,976
Diffusion characteristics	**Facilitated**						11	1			
	Restric./ Mixed						8	0,812	0,322	2,045	0.658
Pial invasion	**No**	5	1				12	1			
	Yes	3	1,151	0,48	2,76	0.752	5	0,608	0,339	1,093	**0.034**
Ependymal extension	**Yes**	8	1				12	1			
	No	5	1,344	1,02	1,771	**0.036**	8	1,519	1,181	1,953	**0.001**
Cortical involvement	**No**	5	1				12	1			
	Yes	1	0,827	0,577	1,186	0.302	5	0,816	0,591	1,126	0.216
Deep white matter invasion	**No**	5	1				11	1			
	Yes	3	1,029	0,536	1,976	0.931	7	1,81	1,123	2,918	**0.015**
nCET crosses midline	**No**	5	1				12	1			
	Yes	2	1,009	0,417	2,441	0.984	5	1,261	0,69	2,307	0.451
CET crosses midline	**No**	5	1				10	1			
	Yes	3	1,623	1,074	2,454	**0.022**	5	1,78	1,219	2,6	**0.003**
Satellites	**No**	6	1				14	1			
	Yes	2	2,581	1,156	5,759	**0.021**	5	1,645	0,959	2,82	0.071

VASARI features that correlated with a worse patient prognosis, included multifocal disease (PFS, p<0.001; OS, p<0.001), solid/nodular contrast enhancement (OS, p=0.035), pial invasion (OS, p=0.034), ependymal extension (PFS, p=0.036 and OS, p=0.001), deep white matter

invasion (OS, p=0.015), bilateral lesion with contrast-enhancement (contrast-enhanced tumor (CET) crosses mid-line) (PFS, p=0.022 and OS, p=0.003), and the presence of satellite lesions (PFS, p=0.021) **(Table VI)**.

3.5. Clinical and imaging factors associated to early clinical deterioration

Early clinical deterioration, prior to adjuvant treatment initiation, was observed in 97 patients (31%), with a consequent decision to forego further adjuvant treatment. Despite a higher rate of tumor biopsy within this subgroup, these patients remarkably represent 17,5% of the surgical cohort and GTR had been achieved in 8,3% (n=2). We therefore explored separately the biopsy and surgery subgroups with early clinical deterioration **(Table VII)**.

Clinical predictors of early deterioration in patients submitted to biopsy included older age (>70, p=0.04, and >75, p=0.009), presentation with gait imbalance (p=0.007) and incapacity for self-care upon presentation (initial KPS <70, p<0.001). MRI predictive features of early deterioration encompassed multifocality (p<0.001), ependymal extension (p=0.026), bilateral contrast-enhancing lesions (p=0.011), presence of satellite lesions (p=0.007) and mild necrosis (p=0.012).

Among the surgical patients (n=31), no patients traits predicted early clinical deterioration. Preoperative MRI with expansive lesions according to T1/FLAIR ratio (p=0.045), with nodular contrast-enhancement (p<0.001) and mild necrosis (p=0.046) were significantly more common in this subgroup of patients. As noted by the postoperative KPS, early deterioration in the surgical group was not attributed to immediate postoperative deterioration **(Table VII)**.

3.6. Comprehensive Multivariate Analysis for Outcome Prediction

As described, univariate analysis identified various clinical and imaging predictors of shorter time to recurrence and overall survival **(Table II and Table VI).** We confirmed that various of these characteristics predict a subgroup of early clinical deterioration, which occurs with

tumor biopsy and surgical resection. Thus, we decided to evaluate these features in a combined (clinical, imaging and treatment features) multivariate model **(Table VIII).**

Table VII. Characteristics of the early clinical deterioration subgroup.

	GBM series	Early clinical deterioration - biopsy subgroup		Early clinical deterioration - surgery subgroup	
	n (%)	n (%)	p-value	n (%)	p-value
	313 (100%)	66 (21%)		31 (9,9%)	
Presentation					
Behaviour	66 (20,8%)	17 (25,8%)	0.296	8 (25,8%)	0.499
Focal deficit	110 (35,6%)	26 (39,4%)	0.417	11 (35,5%)	0.967
Seizures	54 (17,3%)	6 (9,1%)	**0.048**	5 (16,1%)	0.862
Gait	14 (4,5%)	7 (10,6%)	**0.007**	2 (6,5%)	0.576
Headache	65 (21%)	10 (15,2%)	0.207	5 (16,1%)	0.504
Age > 65	140 (44,7%)	33 (50%)	0.332	15 (48,4%)	0.666
Age > 70	83 (26,5%)	26 (39,4%)	**0.004**	10 (32,3%)	0.368
Age > 75	35 (11,2%)	13 (19,7%)	**0.009**	6 (19,4%)	0.109
KPS preop <70	38 (12,1%)	29 (43,9%)	**<0.001**	4 (12,9%)	0.139
MRI features					
Major axis (mean in cm)	6,18	6,78	0,189	6,496	0,647
Eloquent	124 (39,6%)	25 (37,9%)	0,745	12 (38,7%)	0,913
Deep lesions	15 (4,8%)	5 (7,6%)	0,235	2 (6,5%)	0,650
Multifocal	76 (24,3%)	35 (53%)	**<0,001**	8 (25,8%)	0,835
Ependymal extension	89 (28,4%)	26 (39,4%)	**0,026**	6 (19,4%)	0,238
Cysts	49 (15,7%)	3 (4,5%)	**0,020**	2 (6,5%)	0,248
Proportion of necrosis >33%	180 (57,5%)	29 (43,9%)	**0,032**	12 (38,7%)	**0,046**
Satellite lesions	76 (24,2%)	35 (53%)	**0,007**	8 (25,8)%	0,966
T1/FLAIR ratio - expansive	145 (46,3%)	27 (40,9%)	0,432	21 (67,7%)	**0,046**
Thickness of enhancing margin - nodular	60 (19,2%)	15 (22,7%)	0,722	9 (29%)	**<0.001**
CET crosses midline	38 (12,1%)	14 (21,2%)	**0,011**	5 (16,1%)	0.474
KPS posop <70	49 (15,7%)	25 (37,9%)	**<0,001**	2 (3%)	0,552

Clinical presentation (focal deficits, p=0.011, and behaviour alterations, p=0.040) and age (>75, p=0.043) were identified as clinical predictors for shorter time to recurrence. These 3 characteristics doubled the risk of an earlier recurrence. Similarly, 5 predictors for worse overall survival were identified, independent from treatment characteristics. Specifically,

patients older than 75 years old had 2,5-fold (p=0.002) the risk of death, as did presentation with gait imbalance have 3,5-fold higher risk (p=0.025). MRI features that independently predicted a worse overall survival included multifocality (p=0.003), nodular/solid enhancing (p=0.0041) and bilateral contrast-enhancing lesion (p=0.016) **(Table VIII)**.

Table VIII. Multivariate Analysis.

		Progression Free Survival				Overall Survival			
		HR	95,0% CI		p-value	HR	95,0% CI		p-value
			Inferior	Superior			Inferior	Superior	
Age	**<75**	1				1		1	
	>75	2,004	1,022	3,932	**0.043**	2,511	1,413	4,461	**0.002**
KPS	**90-100**	1				1			
	80	1,295	0,74	2,269	0.365	1,367	0,846	2,211	0.202
	70	1,054	0,515	2,156	0.885	1,499	0,825	2,727	0.184
	<70	0,874	0,366	2,088	0.761	1,389	0,676	2,853	0.371
Presentation	**Seizures**	1				1			
	Focal	2,563	1,244	5,28	**0.011**	1,618	0,891	2,935	0.114
	Gait	3,783	0,954	14,998	0.058	3,587	1,173	10,969	**0.025**
	Headache	2,758	1,251	6,08	0,072	1,818	0,958	3,447	0.067
	Behaviour	2,262	1,04	4,923	**0.040**	1,673	0,919	3,045	0.092
Multifocal	**No**	1				1			
	Yes	1,677	0,919	3,061	0.092	2,189	1,307	3,665	**0.003**
Thickness of enhancing margin	**Thin**	1				1			
	Thick/Nodular/ Solid	1,081	0,647	1,804	0.767	2,22	1,032	4,776	**0.041**
Ependymal extension	**No**	1				1			
	Yes	1,146	0,656	2,004	0.633	1,038	0,61	1,765	0.891
CET crosses midline	**No**	1				1			
	Yes	1,077	0,575	2,021	0.816	1,72	1,108	2,671	**0.016**
Surgery	**Surgery**	1				1			
	Biopsy	1,794	1,077	2,989	**0.025**	2,13	1,417	3,201	**<0.001**
Treatment	**Yes**	1				1			
	No	13,503	6,295	28,966	**<0.001**	4,634	2,939	7,309	**<0.001**

4. Discussion

GBM is the most common malignant primary brain tumor and associates with one of the worst 5-year survival rates among all human cancers (23). The grim prognosis associated with this tumor remains similar despite major developments in diagnostic modalities, surgical

techniques and adjuvant treatment strategies (1,24). Apart from molecular markers, various clinical characteristics have been explored as putative predictors of disease outcome. Nonetheless, there is still a significant uncertainty regarding how these predictors should inform clinical decision-making.

Herein, we present the experience of a tertiary hospital with an influence of 591.000 people. All patients included in our series had surgical resection or tumor biopsy with diagnostic intent, in order to undergo adjuvant therapies. Our survival statistics are well within the described in the literature (1,24,25), including the survival benefit observed in the subgroup that undergoes surgery followed by adjuvant chemotherapy and radiotherapy and the value of extent of resection (2,14,15,26).

Our results suggest that, upon patient evaluation, several characteristics are already indicative of distinct patient outcomes. Univariate analysis of initial clinical features evidences that increasing age, presentation with focal deficits, behavior alterations or gait imbalance, as well as low autonomy scores (KPS ≤ 70), anticipate a worse PFS and OS. Similarly, characterization of initial MRI through documentation of VASARI features identified 4 imaging characteristics correlated with poorer PFS (multifocal disease, ependymal extension, bilateral contrast-enhancing lesion and satellite lesions) and 6 features correlated with poorer OS (multifocality, thickness of enhancing margin, pial invasion, ependymal extension, deep white matter invasion and bilateral contrast-enhancing lesion). The presence of cysts was the unique characteristic associated with improved patient outcome, which is consistent with data from a recent meta-analysis (27).

The majority of literature regarding initial MRI features focuses on the preoperative differential diagnosis with lymphoma or brain metastasis, as well as tumor grade prediction. Our study is added to a collective effort to stratify initial patient prognosis through preoperative MRI features. To this end, VASARI characteristics are an advantage, as they are easily obtained and highly standardized. Furthermore, we identified unique MRI features linked to early patient deterioration. Other studies have correlated MRI features with patient prognosis and GBM

biology. Features such as deep white matter invasion and bilateral contrast-enhancing lesions relate to the tumor invasiveness capacity, while the quality/thickness of the contrast-enhancing lesion and necrosis quality report to tumor local aggressiveness (28–30). The presence of satellite lesions or multifocality also probably reflect a more aggressive and advanced tumor, and, especially the latter, have been consistently associated with patient prognosis (31–33).

Taken together, along with the identification of clinical prognosticators, our study reveals 3 main conclusions. The reciprocity between MRI features, clinical presentation and initial functional status suggests that these characteristics frequently reflect tumor biology, and should therefore be considered as indirect markers of disease. For instance, poor KPS (<70) more frequently associated with deep-seated lesions, bilateral contrast-enhancing lesions and multifocality, rather than tumor size/major axis or eloquent location. This denotes that low performance scores frequently reflects patient characteristics and tumor intrinsic biology, including its aggressiveness and ability to induce non-focal cerebral dysfunction. Similarly, presentation with seizures is presented here as a good prognosis marker and has been elsewhere strongly associated with tumor biology (5,26). Thus, these results support that a set of MRI and clinical traits improves surgical decision, especially in particular cases. Further validation of these features in prognostic GBM models and validation in large GBM series is entailed.

Secondly, clinical decision in the elderly patients should be carefully considered in a case-by-case basis. In our study, evaluation of survival data on this population shows a consistent and significant decrease in the survival statistics, although minimal differences are observed regarding clinical presentation, initial performance status or MRI features. In one hand, we documented a higher percentage of biopsies in the elderly patients. We recognize that this result likely reflects the tendency for under-treatment in this population, which is a pertinent issue to be addressed, as there is still ongoing debate regarding the best course of treatment for the elderly patients. Evidence arguing in favor of craniotomy over tumor biopsy in patients

older than 65 years old has increased in the last years and is well documented (34–41). Recent studies further pointed to a significant impact for EOR in the elderly, as GTR led to longer median PFS and OS when compared to subtotal resection (37,39,42,43). In addition, published literature shows that although a general worse prognosis is documented for patients older than 65, there is a subgroup of patients with a remarkable favorable prognosis. Frequently, this subgroup underwent GTR, kept a good functional status and proceeded to adjuvant chemotherapy + radiotherapy (QTRT) (37,42). Our results also identified a subgroup of older patients with longer survival after surgery and QTRT. On the other hand, it remains unclear how to discern which elderly patients will likely benefit from this course of aggressive treatment. The majority of published studies, as well as ours, report a survival benefit in small subgroups of patients. Data suggesting higher complications rates in the older age exists (44,45) and, most importantly, we show higher levels of autonomy loss in the postoperative period in older age groups. This culminates in a significantly lower percentage of elderly patients initiating adjuvant treatments, even when considering those submitted to tumor surgical resection. Taken together, our data most likely reflect the increase frailty of this subgroup which is frequently unaccounted for in the initial evaluation. The Charlson Comorbidity Index has been evaluated as a predictor of outcome in various series, including some in older GBM patients, with incongruent results (42,46,47). Importantly, although we have not included patient comorbidities in our analysis, we concur that a collective effort to define a comprehensive geriatric score, or validate published ones, is of utmost clinical relevance (48,49). We emphasize that while initial functional independency (KPS≥70) is a predictor of better outcome in our series, it did not reach statistical significance when considering the elderly population. This could be explained by the dubious evaluation of patient performance status in older age, as the measure of active work in patients >65 years old becomes more subjective. Considering the nuances of the elderly population, we highlight the importance of thoughtfully consider the presence of MRI features associated with a worse prognosis. In addition, MGMT methylation evaluation before the decision to undergo surgical

resection is another idea in these patients, as it readily identifies the group of patients more likely to benefit from adjuvant treatments. These are prominent questions, as finding the delicate balance against ageism while integrating the inevitable increased frailty of elder patients is crucial. In summary, advanced age alone should not preclude surgery followed by adjuvant treatments. However, careful individual evaluation of these patients must consider characteristics that point to an early deterioration, not only as a measure of survival, but also as a reflection of rapid loss of autonomy and, potentially, quality of life. Indeed, due to the retrospective nature of our series we unfortunately could not include include quality of life scores in this study and aim to include this outcome in future studies.

Lastly, one of our most striking results is the percentage of patients who did not initiate adjuvant treatment after diagnosis (n= 97, 31%) due to early clinical deterioration. These include 31 patients that had been submitted to surgical resection, which consists of 17,5% of the surgical cohort. Early clinical deterioration is not unique to our series (50). It importantly reflects the individual variability within the survival statistics for GBM, as well as the unmet task of refining survival prognostication prior to treatment decision. We characterized this subgroup of patients, identified its clinical and imaging markers, and conclude that these patients are more frequently older patients, with lower performance status and MRI features indicative of tumor infiltration and invasiveness. In patients submitted to surgery, early clinical deterioration was observed more frequently in those which exhibited nodular lesions with avid contrast-enhancement, mild necrosis and mild infiltration/edema (expansive lesions), which may point to tumors with an increased biological growth rate and aggressiveness. Further studies on this topic should aim to confirm MRI features and clinical characteristics, which together with the evaluation of patient frailty, will allow the identification of a subgroup highly likely to experience a poor outcome in the short term. On the meanwhile, it is important to consider that surgical consent and information provided to the families of patients with expected worse prognosis, should include the fact that tumor biopsy and diagnosis do not

guarantee nor adjuvant treatment initiation nor an alteration to the natural course of the disease.

Our study is inherently limited by its retrospective design, including the necessity to retrieve data from clinical records. In addition, MRI scans were acquired at various hospitals. It represents a series with an important casuistic of biopsied patients, whose characteristics are in concordance with decision algorithms observed in high-volume centers and previously reported (51,52). Although IDH status was not evaluated for the whole cohort, it did not influence the analysis in the subset of 135 patients with known IDH status. Despite these limitations, our comprehensive analysis of several clinical and MRI characteristics establishes the possibility of preoperative patient survival prognostication, and hence clinical decision.

5. Conclusions

Age, KPS and clinical presentation reflect patient autonomy, frailty and, frequently, underlying tumor characteristics. These clinical characteristics should be complemented with MRI features in order to improve survival prognostication, especially in particular cases.

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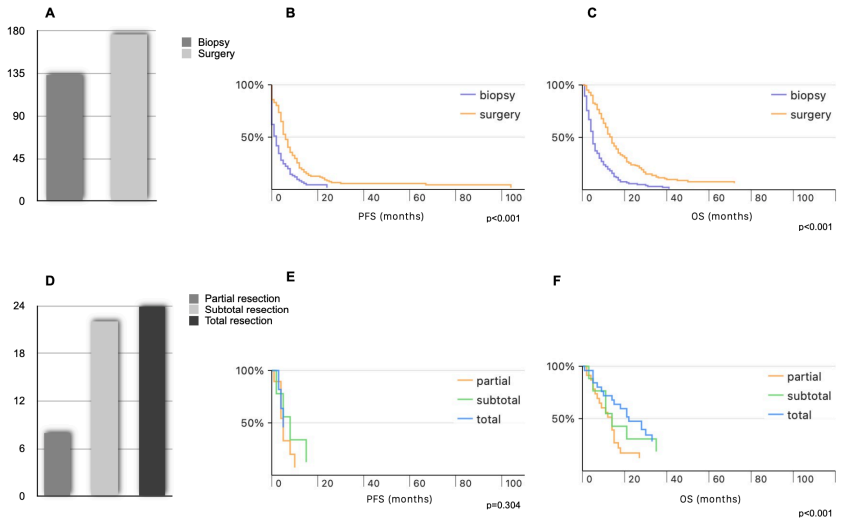
7. Supplementary data



Supplementary Figure 1. Surgical treatment characteristics and outcomes. Tumor biopsy was performed in 134 patients (43,5%) **(1A)** and determined a worse PFS and OS survival **(1B and 1C)**. In addition, in patients with postoperative MRI, EOR was determined **(1D)** and higher EOR with GTR associate with longer OS **(1F)**.

Supplementary Table I. Characteristics associated to tumor biopsy vs surgical resection

	GBM series	Biopsy	Surgery	
	n (%)	n (%)	n (%)	p-value
	313 (100%)	136 (43,5%)	177 (56,5%)	
Age >70	80 (25,6%)	42 (31,3%)	17 (17,5%)	**0,042**
KPS <70	38 (12,1%)	25 (18,7%)	4 (4,1%)	**0,002**
Motor deficits	53 (16,9%)	32 (23,9%)	21 (11,7%)	**0.005**
Behaviour changes	65 (20,8%)	28 (20,9%)	37 (20,7%)	0,961
Focal	116 (37,1%)	57 (42,5%)	59 (33%)	0,083
Headache	74 (23,6%)	26 (19,4%)	48 (26,8%)	0,127
Seizure	54 (17,3%)	21 (15,7%)	33 (18,4%)	0,522
Multifocal	76 (24,3%)	56 (41,8%)	20 (11,2%)	**<0,001**
Ependymal extension	89 (28,4%)	54 (40,3%)	35 (19,6%)	**<0,001**
CET crosses midline	38 (12,1%)	24 (17,9%)	14 (7,8%)	**0.007**
Eloquent location	124 (39,6%)	56 (41,8%)	68 (38%)	0,496
Location				
Frontal	112 (36,1%)	48 (35,8%)	64 (35,8%)	0,99
Temporal	96 (30,4%)	32 (23,9%)	64 (35,8%)	**0,024**
Deep	15 (4,8%)	13 (9,7%)	2 (1,1%)	**<0,001**

CHAPTER IV: PD-L1 tumor expression is associated with poor prognosis and systemic immunosuppression in glioblastoma[i]

[i] Noronha C, Ribeiro AS, Taipa R, Leitão D, Schmitt F, Reis J, Faria C, Paredes J. PD-L1 tumor expression is associated with poor prognosis and systemic immunosuppression in glioblastoma. J Neurooncol 156(3): 453-464, 2022

PD-L1 tumor expression is associated with poor prognosis and systemic immunosuppression in glioblastoma

1. Introduction

Glioblastoma is the most aggressive form of gliomas, a group of primary tumors arising from glia or their precursors within the central nervous system. Despite their low incidence, these highly aggressive tumors encase a disproportionate rate of cancer-related mortality, with a 5-year overall survival of only 6.8% [1, 2]. The standard of care remains surgical resection followed by radiotherapy and chemotherapy, usually temozolomide [2]. Glioblastoma represents one of the biggest therapeutic quests in current oncology.

The development of effective treatments for glioblastoma is challenged by its intracranial location, rapid progression, infiltrative growth and extensive intratumoral heterogeneity. In addition, glioblastoma is one of the most immunosuppressive solid tumors [3], capable of inducing early systemic T-cell dysfunction and escape from immunosurveillance [4-6]. Indeed, glioblastoma patients frequently exhibit severe lymphocytopenia comparable to AIDS-level CD4 counts, which has been shown to portend a worse prognosis [7, 8]. In parallel to systemic immunosuppression, inflammation has been thoroughly related to cancer initiation and progression [9, 10]. Inflammatory parameters, such as neutrophil-to-lymphocyte ratio (NLR), have been shown to reflect prognosis in various tumors [11, 12]. These results have been validated in glioma cohorts and an elevated NLR in peripheral blood has been suggested to reflect a worse patient prognosis [13, 14].

Apart from the systemic inflammation and immunosuppression, glioblastoma induces a significant local immunosuppression. Mechanisms of immunosurveillance escape include tumor expression of PD-L1, a negative regulator of lymphocyte activation and so-called immune checkpoint, which interacts with PD-1 expressed by activated CD4+ or CD8+ T-lymphocytes [15]. Antibodies that block the immune checkpoints have demonstrated activity

in a variety of solid tumors, and are now standard of care for instance in brain-metastasized melanoma [16]. Several biomarkers of response to immune-checkpoint inhibitors have been identified and disputed, such as mutational burden [17, 18], DNA-repair deficiency [19], and checkpoint-ligand expression [20].

Amid the successes of immunotherapy in several tumor types, results for glioblastoma unfortunately suggest this tumor as an epitome of resistance to immunotherapy. Results for immune-checkpoint inhibition in newly diagnosed glioblastomas failed to show a benefit for the addition of nivolumab to radiation (CheckMate-498) [21] or radiation and temozolomide in MGMT-methylated patients (CheckMate-548) [22] when compared to radiation plus temozolomide. In recurrent glioblastoma, nivolumab failed to be superior to bevacizumab (CheckMate-143) [23]. In this particular clinical trial, only 7.8% of patients exhibited response to nivolumab compared to the 23.1% of patients with response to bevacizumab. The median duration of response to nivolumab, however, was a striking median of 11.1 months compared to 5.3 months for bevacizumab. These results suggest that patient selection might play a key role in the efficacy of PD-1 blockade for glioblastoma. Additional studies focused on identifying markers for glioblastoma response to immune-checkpoint inhibitors, pointing to a complex interaction by genomic and transcriptomic characteristics (such as PTEN and BRAF mutations) with the dynamic composition of the tumor microenvironment [24-26].

The notorious glioblastoma intratumoral heterogeneity urges for the identification of biomarkers that do not merely reflect its molecular characteristics, but mostly its dynamic evolution. We thus characterized PD-L1 expression in a large and well characterized glioblastoma patients' cohort, which includes paired recurrent samples, and aimed to establish patterns of expression associated to patient characteristics, systemic immunosuppression and inflammation, tumor molecular and neuropathological characteristics, including the presence of tumor infiltrating lymphocytes (TIL).

2. Materials and Methods

2.1. Human glioblastoma samples

A retrospective series of 313 patients who underwent biopsy or surgical resection of newly diagnosed glioblastoma (GBM), between 2010 and 2017, at the Neurosurgery Department, Hospital de Santo António - Centro Hospitalar Universitário do Porto, was included in the study. Formalin-fixed and paraffin-embedded (FFPE) tumor tissues were histologically analyzed for all primary tumors, as well as for matched local recurrences (39 patients). Clinical data, treatment decision and patient survival were extracted by chart review for all the cases included in the series. Initial full blood count data was collected and documented, data from 3 patients was excluded due to previous corticoid therapy. Lymphocytopenia was considered when lymphocyte count was inferior to 1000/µL. NLR (neutrophil -to-lymphocyte ratio) was calculated as absolute neutrophil count divided by absolute lymphocyte count. Initial MRI was also evaluated, and imaging characteristics documented. Information regarding IDH1 (R132H) and ATRX mutations was available in 139 patients.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration. Moreover, it has been also approved by the ethics committee at Centro Hospitalar Universitário do Porto (2017-104 (093-DEFI/091-CES).

2.2. Neuropathological and tumor infiltrating lymphocyte evaluation

Neuropathological characteristics were analyzed in detail in hematoxylin and eosin staining from the initial tumor samples, including: necrosis presence and type (coagulative, pseudopalisade); thrombosed or glomeruloid vessels; mitosis; myxoid stroma; calcifications;

papillary structures; oligodendroglial, gemistocytic, sarcomatous, epithelial and glial components. Tumor infiltrating lymphocyte (TIL) density was evaluated semi-quantitatively according to previously published data [27] into sparse, moderate or dense. In addition, TIL location was documented into 4 subsets: within glioblastoma tissue, perivascular region, perinecrotic region and brain infiltration zone [28].

2.3. PD-L1 Immunohistochemistry

Immunohistochemical staining for PD-L1 was performed in 3µm sections using a mouse monoclonal antibody (clone 22C3, Dako), previously tested [29]. The assay was carried out on an automated immunostaining system (Ventana Benchmark Ultra, using the OptiView DAB IDH Detection kit, according to manufacturer's instructions). Human placenta has been used as a positive control and performed in parallel with FFPE sections from the GBM series. Positivity was considered membranous staining ≥ 5% in tumor cells [20].

2.4. Statistical Analysis

Statistical analyses were performed using the computer software package SPSS (15.0, IBM). Chi-square test was used to assess group differences as appropriate. Overall survival from diagnosis of glioblastoma to death or last follow-up was estimated using the Kaplan-Meier limit method. The log-rank test was used to assess group differences. A 2-tailed significance level (p value) of 0.05 was applied.

3. Results

3.1. Association between GBM patients' initial lymphocytopenia and NLR with progression-free and overall survival

Half of the patients were lymphocytopenic upon tumor diagnosis (n=167, 53,4%), uniformly distributed irrespective of age, gender and functional status. The presence of lymphocytopenia at diagnosis was significantly associated with a shorter progression free survival and overall survival (log rank test, p=0.03 and p=0.022, respectively) **(Figure 1A and 1B)**.

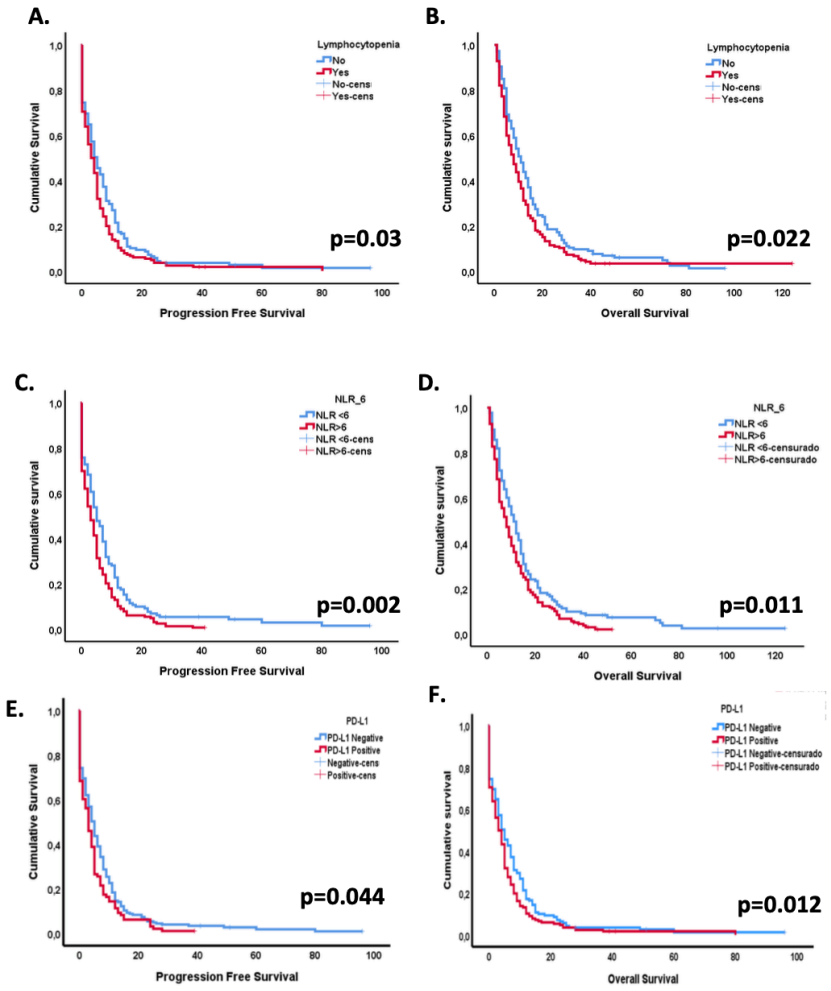


Figure 1. Inflammation and immunosuppression cues in GBM. Association between GBM patient's initial lymphocytopenia **(A,B)**, NLR **(C,D)** and PD-L1 expression **(E,F)** with progression free survival and overall survival.

Concerning the NLR, the mean value in our cohort was 9.654. Interestingly, IDH1 and ATRX mutations had a lower NLR mean of 3.953 and 7.18, respectively. Higher NLR was also significantly associated with a shorter time to tumor recurrence (log rank test, p=0.010) and shorter overall survival (log rank test, p=0.020) **(Figure 1C and 1D)**. Interestingly, a cut-off value of 6 for NLR was found to stratify patient overall survival in a significant way (log rank test 12 vs 8 months, p=0.011).

3.2. Neuropathological evaluation and TIL infiltration

Relevant histologic features of primary tumors are summarized in **Table I**. A glial component was observed in 98,1% of the tumors, being the most proliferative component in most tumors. A sarcomatous component was evident in 18,2% and an epithelial component was identified in 13,4%. Positivity for IDH1 mutation was observed in 5% of patients and in 17% of patients for ATRX mutation.

Table I. H&E evaluation of neuropathological features.

			n	%	OS (months)	p-value
Tissue components		**Glial**	307/313	98,1	11	**0.0462**
		Sarcomatous	57/313	18,21	8	
		Epithelial	42/313	13,4	10	
TIL	**Density**	**Sparse**	53/82	64,6	12	0.096
		Moderate	22/82	26,8	12	
		Dense	07/82	8,5	8	
		Very Dense	-			
	Location	**Perivascular**	41/82	50	12	0.086
		Perinecrotic	7/82	8,5	12,5	
		Within tumor tissue	10/82	12,1	18,5	
		Tumor periphery	24/82	29,2	8	

A total of 82 (26,2%) tumors showed TIL of variable density. Sparse-to-moderate TIL were preponderant (91,4%), with a minority of cases showing dense infiltration (8,5%). **Table I** details semi-quantitative evaluation of TIL density. These were predominantly localized in perivascular areas (50%) and tumor infiltrative zones (30,4%) and rarely showed a peri-necrotic location (8,5%). A tendency for worse overall survival for TIL location in tumor periphery was identified (8 months vs 12 months in perivascular location and 18,5 months within the tumor, p=0.086). Interestingly, TIL were significantly more frequent in tumors with a sarcomatous component (55,9% vs 19.3%, chi-square, p<0.001). No differences in patients' survival was observed regarding TIL infiltration subgroups, including TIL presence, density or location.

3.3. PD-L1 expression in primary and recurrent glioblastoma

PD-L1 staining of variable extent was found in 31% (n=98) of the tumor samples **(Supplementary Table I and Figure 2)**. In the majority of the patients, a granular membranous staining was found in sparse tumor cells. PD-L1 positivity in over 20% of the tumor was found in about 40% of positive cases, from which 8% showed staining in more than half of the tumor. Contiguous infiltrated or preserved brain parenchyma was evaluated when present, and a faint diffuse staining was identified in a minority of the samples (<10%).

Matched primary and recurrent samples were obtained in 39 cases. Curiously, PD-L1 expression was higher in recurrent cases, in up to 46% of the samples. The primary tumor was negative for PD-L1 in 29 cases and positive in 10 cases. The majority of negative cases remained negative (n=19), while there was gain of PD-L1 expression in tumor recurrence for 10 cases (34%). Of the 10 primary PD-L1 positive tumors, 8 maintained positivity and 2 cases lost PD-L1 staining.

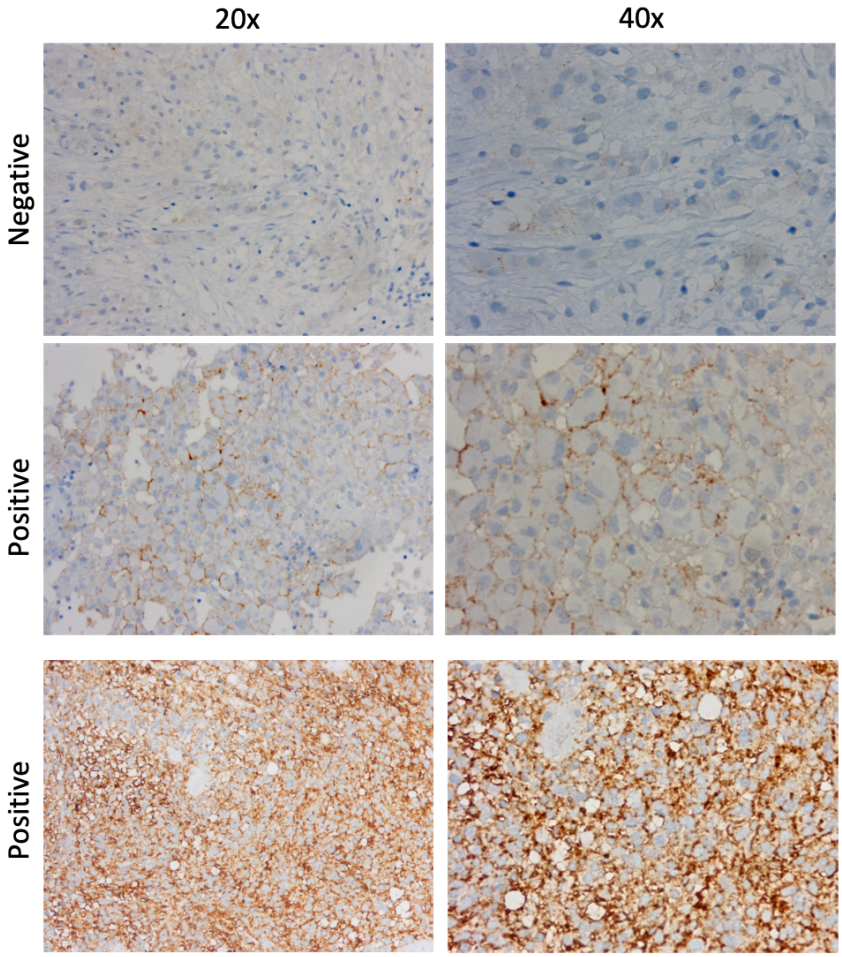


Figure 2. Representative immunohistochemistry images for PD-L1 expression. 20x and 40x magnification.

3.4. Association between PD-L1 expression and clinico-pathological characteristics

Relevant associations between PD-L1 expression and clinico-pathological characteristics are summarized in **Table II**.

Patient age, gender, tumor location or multifocality did not correlate to tumor PD-L1 expression. There was also no correlation between preoperative treatment with corticosteroids and the expression of this biomarker. However, lower lymphocyte count on peripheral blood significantly correlated with PD-L1 positivity (chi-square, p=0.018), as did a higher (>6) initial NLR (chi-square, p=0.002). PD-L1 expression was common in tumors with

lymphocytic infiltrates (chi-square, p<0.001), in particular with dense (72%) to moderate (83,3%) density.

Table II. Correlation between PD-L1 expression and clinicopathological characteristics.

		PD-L1 expression		
		Negative (n= 215)	Positive (n=98)	p-value
		n	n	
Lymph infiltrates/ Inflammation	**No**	177	54	**<0.001**
	Yes	38	44	
Lymph infiltrates/ Inflammation Density	**No**	177	54	**<0.001**
	Sparse	33	21	
	Moderate	4	18	
	Dense	1	5	
Lymph infiltrates/ Inflammation Location	**No**	177	54	**<0.001**
	periphery	3	21	
	perivascular	21	20	
	perinecrotic	4	3	
	tumor	10	0	
Lymphocytopenia	**No**	110	36	**0.018**
	Yes	105	62	
IDH	**No**	89	43	0.69
	Yes	7	0	
ATRX	**No**	75	40	**0.032**
	Yes	21	3	
Sarcomatous component	**Negative**	192	62	**<0.001**
	Positive	23	36	
NLR	**NLR <6**	104	29	**0.004**
	NLR>6	111	69	

Moreover, PD-L1 expression was significantly associated with TIL location (chi-square, p<0.001), most commonly found at tumor periphery (86,4%), compared with perivascular location (39%) or within the tumor tissue (1%). The majority of the tumors with sarcomatous features showed positivity for membranous PD-L1 (54,2%, chi-square, p<0.001). Although not significant, tumors with IDH mutation were all negative for PD-L1 expression and less than 10% of patients with ATRX mutation showed PD-L1 expression.

3.5. Association between PD-L1 expression and patient's prognosis in glioblastoma

The established prognostic factors for GBM were validated in our cohort, including age (log rank test, p=0.001), initial functional status (log rank test, p=0.001), extent of resection (EOR) (log rank test, p<0.001), complimentary treatment (log rank test, p<0.001), IDH (log rank test, p=0.001) and ATRX (log rank test, p<0.001) mutations **(Supplementary Table II).** Tumor membranous expression of PD-L1 in the primary tumor also significantly correlated to shorter time to recurrence (log rank test, p=0.044) and worse overall survival (log rank test, p=0.012) **(Supplementary Table II, Fig. 1E-F)**. Tumor PD-L1 expression showed a survival disadvantage for all treatment modalities, namely RT (log rank test, p<0.001), sequential RT+QT (log rank test, p=0.025), and Stupp protocol (log rank test, p=0.008). Strikingly, in patients who did not received treatment following tumor biopsy or surgical resection, the survival disadvantage associated to PD-L1 expression was not observed (log rank test, p=0.418). Although an increase in the percentage of positive cases was identified upon tumor recurrence, no significant correlation between immunostaining for PD-L1 in recurrent cases and patient prognosis was found. Importantly, on multivariate analysis, we identified PD-L1 expression in the primary tumor as an independent predictor of a worse prognosis **(Table III)**.

Table III. Multivariate analysis for classical prognostic factors and PD-L1.

		Progression free survival		Overall survival	
		HR (95% CI)	p value	HR (95% CI)	p value
Age	**< 60 years**	1		1	
	> 60 years	1.471 (1,065-2,032)	**0,019**	1.734 (1,256-2,393)	**0,001**
KPS	**< 70**	1		1	
	> 70	0,742 (0,186–1,063)	0,103	0,648 (0,453–0,928)	**0,018**
Extent of resection	**Biopsy**	1		1	
	Partial	0,767 (0,385-1,525)	0,449	0,871 (0,436-1,737)	0,695
	Subtotal	0,754 (0,502-1,132)	0,173	0,636 (0,428-0,946)	**0,026**
	Total	0,511 (0,335–0,779)	**0,002**	0,400 (0,260-0,614)	**0,000**
Adjuvant Treatment	**No**	1		1	
	Yes	0,175 (0,112-0,276)	**0,000**	0,911 (0,212-0,458)	**0,000**
PD-L1	**Negative**	1		1	
	Positive	1.385 (0,995–1,927)	0,053	1.660 (1,190–2,315)	**0,003**

4. Discussion

PD-1/PD-L1 interactions are considered key to the immunological control of cancer and have been extensively explored in melanoma and epithelial cancers [30, 31]. Nevertheless, the rate of PD-L1-positive immunohistochemistry cases, correlation to clinical prognosis, and response to immunotherapy remains overall disputed [32-48].Our main goal was to evaluate PD-L1 expression in a large series of glioblastoma patients, with over 300 primary tumors, while using 39 matched recurrences to document PD-L1 expression upon tumor recurrence. In our cohort, up to 31% of tumors showed PD-L1 staining, of which 20% had staining of more than 40% of tumor cells and 8.2% more than 50% of the tumor. A remarkable increase in staining in recurrent disease was identified, in up to 46% of the tumors. Furthermore, 36% of initial PD-L1 negative cases showed matched PD-L1 positive recurrent disease. These results support similar data suggesting a conjectural increase in PD-L1 expression as a mechanism associated to treatment resistance [49]. Furthermore, subgroup analysis by treatment

modality curiously showed that, contrary to all treatment modalities, PD-L1 expression did not influence prognosis in treatment-naive patients. This data adds to the hypothesis that PD-L1-associated patient prognosis may mostly reflect treatment resistance mechanisms, as it foremost seems to impair the expected benefit of complementary treatments. We should, however, emphasize the retrospective nature of our study and further studies are needed in order to understand the dynamic expression of PD-L1 in the context of tumor local immunosuppression, as well as its role in therapy resistance.

Nevertheless, our patients' survival analysis correlated PD-L1 positivity with a worse progression free and overall survival, further identifying PD-L1 expression as an independent prognosis factor in glioblastoma. This data concurs with similar series in the literature [50, 51] and, taken together, highlights the importance of the PD-1/PD-L1 axis in glioblastoma pathogenesis. In addition to the potential direct effect of an immunosuppressive environment in patient prognosis; a putative relationship between the presence of tumor immune infiltrates, expression of immunosuppressive molecules and tumor subtype has recently gained rising importance. Taken together, recent studies propose disease-specific immune profiles, dependent on glioblastoma subtypes [52-54] with a consistent TIL-enrichment associated to a mesenchymal phenotype [25, 52, 54]. Indeed, PD-L1 positivity in our cohort was more frequent in IDH and ATRX wild-type tumors with areas of sarcomatous dedifferentiation. These results are in unison with reports correlating PD-L1 expression with the mesenchymal subtype in glioblastoma [28, 52, 55-57] as well as with data in systemic tumors, suggesting a relationship between tumor immune escape and the ability to undergo an EMT process. Tumor resistance to immune degradation may thus be, either or both, an inductor of a mesenchymal phenotype or perpetuated by the activation of EMT underlying pathways.

Other mechanisms have been proposed for glioblastoma local immunosuppressive environment, including cell surface factors, cytokine secretion/dysregulation, myeloid and lymphocyte dysfunction [58]. Initial case reports and small series have encouraged

immunotherapy for glioblastoma [59-61], but results published to date on large clinical trials have failed to show a clinical efficacy [21, 23] and seemingly suggest that perhaps only a minority of glioblastoma patients benefit from these treatment strategies. Results on neoadjuvant treatment with pembrolizumab in recurrent glioblastoma demonstrated a significantly improved overall survival, suggesting that timing and association with other treatments should be explored [62]. In addition, a pivotal focus on T-cell dysfunction pairs the growing interest and applicability of immunotherapy to treat glioblastoma. Tumor immune infiltrates have been heterogeneously correlated to tumor prognosis in a variety of cancers [63, 64] This is partly explained by their diverse composition and functionality, as well as localization [65, 66]. Initial reports documented TIL presence in 28-60% of gliomas. Similar to other tumors, subsequent data on gliomas remains incongruous regarding TIL incidence, degree of infiltration and location (review by [67]). Absence of recent and consistent literature prevents an assertive correlation between glioma TIL and prognosis. In our cohort, TIL infiltrates were identified in a minority (26%) of tumors. These were more commonly sparse-to-moderate in density and located in perivascular areas or tumor periphery, rarely within tumor tissue. TIL presence and density did not correlate with patient prognosis. However, we identified a tendency for worse overall survival considering TIL location in tumor periphery. We interpret this result considering the so-called immune-excluded phenotype, in which T cell accumulate in tumor margins unable to invade the malignant tumor core [66]. Curiously, in our cohort, TIL location in tumor periphery was associated with PD-L1 expression in 21 of the 24 cases (87,5%) suggesting that a mechanism, perhaps the ability of tumor to induce T-cell dysfunction, may regulate T cell location.

Apart from a profoundly local immunosuppressive environment, glioblastoma is capable of inducing a severe systemic immunosuppression, in particular with a tendency to cause systemic inflammation and severe systemic T cell deficits [4, 5, 68]. Consequently, we explored two easily accessible and cost-effective markers of systemic inflammation and immunosuppression - initial lymphocyte count and NLR. We found that, early upon diagnosis,

the majority of patients showed lymphocytopenia and high NLR, and that these two markers identified patients with a worse overall survival. In fact, NLR has consistently been described as an important factor for disease prognosis in glioblastoma and other systemic cancers [14]. However, how exactly a high NLR reflects tumor pathogenesis and, thus, prognosis is less clear: it may merely reflect a relative lymphocytopenia or neutrophilia; or, on the other hand, it may evidently identify an abnormally coordinated inflammatory and immune response. Low T cell counts have been previously conceived as a treatment-induced side effect, however, recent evidence has proposed T cell sequestration in the bone marrow as a mechanism for lymphopenia in glioblastoma, confirming that initial lymphopenia may well be a reflection of glioblastoma-associated systemic immunosuppression [4, 7, 69, 70].

In order to improve treatment efficacy, it is imperious to understand the intrinsic mechanisms associated to glioma immune responses, to characterize glioma subtype-defined immunophenotypes and to evaluate whether these differences lead to a clinically relevant patient stratification. Identification of predictive biomarkers of treatment response is mandatory. In a large cohort of patients, we emphasize the need to clarify the clinical relevance of lymphocytopenia, as well as TIL presence and location. Moreover, our data suggests that both lymphocytopenia and NLR warrant further evaluation as easily accessible and predictive biomarkers, as we speculate these alterations may reflect glioblastoma-induced immunosuppression.

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6. Supplementary data

Supplementary Table I. Semi-quantitative evaluation of PD-L1 expression in glioblastomas.

		Primary		Recurrent	
		n	%	n	%
PD-L1 expression	Negative (<5%)	215	69%	21	53,80%
	Positive (>5%)	98	31%	18	46,20%
	5-10%	60	61,20%	13	72,20%
	20-40%	30	30,60%	3	16,70%
	>50%	8	8,20%	2	11,10%

Supplementary Table II. Characteristics correlated to patient's prognosis in glioblastoma.

		Progression Free Survival					Overall survival				
		Mean	Std Dev.	> 95% CI		p-value	Mean	Std Dev.	> 95% CI		p-value
				Inferior	Superior				Inferior	Superior	
Age	**< 60 y**	9,986	1,926	6,210	13,761	**0,005**	20,797	3,236	14,455	27,139	**0,001**
	> 60 y	5,164	0,743	3,709	6,619		11,525	1,215	9,142	13,907	
KPS	**<70**	4,568	1,844	0,953	8,183	**0,005**	9,864	2,807	4,362	15,366	**0,001**
	>70	7,737	1,032	5,715	9,759		15,808	1,395	13,073	18,543	
EOR	**Biopsy**	2,562	0,368	1,840	3,283	**<0,001**	6,985	0,692	5,628	8,342	**<0,001**
	Partial	6,133	1,588	3,020	9,246		10,875	2,240	6,485	15,265	
	Subtotal	11,277	2,119	7,124	15,431		18,906	2,162	14,668	23,144	
	Total	9,955	1,502	7,012	12,899		20,070	2,582	15,009	25,131	
Adjuvant Treat.	**No**	0,404	0,184	0,044	0,765	**<0,001**	4,622	0,584	3,478	5,766	**<0,001**
	Yes	9,684	0,985	7,753	11,615		18,203	1,427	15,407	20,999	
IDH	**No**	8,215	1,275	5,717	10,714	**0,002**	17,292	2,412	12,564	22,021	**0,001**
	Yes	44,722	8,078	28,890	60,554		60,143	7,644	45,161	75,125	
ATRX	**No**	7,738	1,322	5,147	10,330	**<0,001**	14,284	1,768	10,819	17,750	**<0,001**
	Yes	21,293	5,464	10,584	32,003		41,000	8,593	24,158	57,842	
PD-L1	**Neg**	8,100	0,981	6,177	10,023	**0,044**	16,149	1,459	13,289	19,009	**0,012**
	Pos	5,092	0,709	3,702	6,482		10,673	1,147	8,425	12,922	

CHAPTER V. Cadherin expression profiles define glioblastoma differentiation and patient prognosis[i]

[i] Noronha C, Ribeiro AS, Carvalho R, Mendes N, Reis J, Faria C, Taipa R, Paredes J. Cadherin expression profiles define glioblastoma differentiation and patient prognosis . Cancers, 2024

Cadherin expression profiles define glioblastoma differentiation and patient prognosis

1. Introduction

Cadherins are membranous calcium-dependent proteins crucial for cell-cell adhesion [1] and thus key players in a variety of cellular processes [2, 3]. Most frequently, cadherins assemble into adhesive junctions between cells expressing the same cadherin subtype; however, their expression can also be highly heterogeneous, since cells can express more than one cadherin subtype [4, 5]. Importantly, through their intracellular cytoplasmic tail, cadherins associate with numerous signaling proteins, participating in a dynamic process dependent on the cellular context and which culminates in the regulation of physiologic mechanisms, such as cell proliferation, differentiation, and migration [3, 6-9]. Most likely, qualitative and quantitative differences in the expression of different cadherin subtypes confer tissue-specific characteristics and functional roles [2, 10].

Epithelial (E-), neuronal (N-), and placental (P-) cadherins are classical cadherins, the first members of the superfamily to be identified (CDH1, CDH2, CDH3, respectively). Apart from their important physiological roles [6, 8, 9], these proteins have been highly implicated in oncogenesis. Considering epithelial cancers, the loss of E-cadherin expression is considered a prerequisite for tumor cell invasion and dedifferentiation [11-14] and it frequently parallels with the de novo expression of mesenchymal cadherins, such as N-cadherin, a process called "cadherin switch", which is a marker of the epithelial to mesenchymal transition (EMT) molecular process [15-18]. Nevertheless, evidence from other tumors has questioned this simplified model of cadherins expression in cancer [19-22]. Tissue-specific expression profiles of cadherins suggest that, similarly to its cancer-related functions, cadherin expression subtypes must be context dependent, particularly when considering the added role brought by interactions between these proteins [23]. Interestingly, current knowledge on

the EMT process parallels with this concept of dynamic cadherins expression, with transitory and function-dependent regulation of gene expression, resulting in a complex non-binary program, frequently reaching a hybrid phenotype between epithelial and mesenchymal states [24]. Importantly, the presence of various subclones with distinct epithelial, mesenchymal or intermediate/hybrid states within the same tumor has been observed [25], which strongly contributes to tumor heterogeneity.

In the Central Nervous System (CNS), the role of classical cadherins in gliomas remains to be clarified, as published data remain limited and conflictive [26-29]. Gliomas are primary brain tumors notorious for their invasiveness, diffuse growth and treatment resistance [30, 31]. Glioblastoma (GBM), the most frequent glioma, is a heterogeneous and aggressive tumor, with a median overall survival of 15 months [32, 33]. In 2010, transcriptomic analysis of glioblastoma established the presence of a molecular classification into 4 subtypes: proneural, neural, classical and mesenchymal [34]. Since then, the mesenchymal subtype of glioblastoma has been thoroughly investigated and suggested to associate with an invasive phenotype, increased aggressiveness and robust therapy resistance [35, 36]. However, how the mesenchymal transcription subtype aligns with a phenotypic mesenchymal transition, or how the concept of EMT applies to GBM and to other tumors with non-epithelial origin, is still uncertain. In particular, the canonical EMT-related E- to N-cadherin switch is unlikely to be a central process in these neoplastic diseases and the assumption that N-cadherin expression marks for such a mesenchymal phenotype is likely farfetched. A recent review highlights how our current knowledge about cadherin expression in gliomas arises mostly from small sampled series, with significant contradicting results [29]. For instance, N-cadherin expression has been associated with both: a protective role, with a decrease in tumor migration, invasiveness and dissemination [27, 37-39]; as well as with a detrimental role, with a documented association between increased N-cadherin expression and high pathological glioma grade [40, 41], the presence of radioresistant glioma stem cells [42], and a significant decrease in patient survival [28, 41]. Taken together, published results collectively point to a

broad N-cadherin expression in gliomas, with a likely increase from low grade to high grade gliomas [41, 43]. E-cadherin expression in gliomas is reduced and suggested to be more frequent in low grade gliomas than in GBM [44, 45]. Interestingly, a recent paper studied, for the first time, P-cadherin expression on gliomas and demonstrated increased expression in high grade tumors and a correlation with a decreased overall survival in GBM [46]. Moreover, P-cadherin expression was shown to induce an increased tumorigenic behavior, invasive capacity and stem-like characteristics in GBM cell lines [46].

Overall, conclusions of published results on the biological role of cadherins expression in GBM dedifferentiation, dissemination or disease aggressiveness are thus far mostly contradictory and, as a consequence, highly inconclusive. In order to further clarify this topic, we herein present the analysis of cadherins expression in a large series of GBM and correlate their expression profiles with clinical, imaging and survival data. In addition, we explored cadherins expression interplay and how it could improve the understanding of GBM exquisite unknown pathogenesis.

2. Materials and Methods

2.1. Patients and materials

A retrospective series of 313 patients who underwent biopsy or surgical resection for newly diagnosed glioblastomas (GBM), between 2010 and 2017, at the Neurosurgery Department, Centro Hospitalar de Santo António (Porto, Portugal), has been retrieved. Formalin-fixed and paraffin-embedded (FFPE) tumor tissues were histologically analyzed for all primary tumors, as well as for matched local recurrences in 38 patients. Clinical data, treatment decision, patient and survival follow-up data were extracted by patient's records review for all the cases included in the series. Initial MRI was also evaluated and imaging characteristics documented. This study has been performed in accordance with the national regulative law for the handling

of biological specimens from tumor banks, as well as under the international Helsinki declaration. Moreover, it has also been approved by the ethics committee at Centro Hospitalar de Santo António (DEFI 104-17 (093-DEFI/091-CES).

2.2. Neuropathological evaluation

All cases were reviewed by an experienced neuropathologist, blinded to clinical and demographical details. Neuropathological features were analyzed in hematoxylin and eosin staining from the initial tumor samples. Neuropathological phenotypes were evaluated and the presence of distinct areas of differentiation were documented according to the following features: 1) the glial component, which included agglomerates of fibrillary astrocytes, gemistocytes, multinucleated giant cells or cells with oligodendroglial features; 2) the epithelioid component, which has been considered when morphologic epithelial appearance, with large and polygonal shape cells were present, with abundant cytoplasm and defined cell border; 3) a sarcomatous component was also considered, whenever areas of sarcomatous or mesenchymal differentiation were observed, often comprising chondral or osteochondral elements. The predominant component (>50% of the tumor) was documented as well.

2.3. Immunohistochemical Staining

Immunohistochemical (IHC) staining was performed with specific monoclonal antibodies for the classical cadherins N-, E-, and P-cadherins in 3µm sections: E-cadherin (1:100, rabbit, clone 24E19, Cell Signalling, USA), P-cadherin (1:50, mouse, clone C56, BD Transduction, USA) and N-cadherin (1:100, mouse, clone 3B9, ThermoFisher, USA). Briefly, dewaxing and epitope recovery was performed using Dewax and HIER Buffer M (Epredia, Kalamazoo, USA) and all ER were heat-induced in an Epredia PT Module, according to the standard protocol. The downstream IHC protocol was performed in the Lab VisionTM Autostainer 480S, using the

Epredia UltraVision™ Quanto-HRP Detection System. The time for primary antibody incubation was 60 minutes, with the exception for the anti-P-cadherin antibody, where a 90 minutes incubation was performed. All reactions were revealed with 3,3'-diaminobenzidine (DAB) chromogen. Tissues were then counterstained with HIGHDEF® hematoxylin (Enzo, Farmingdale, USA), dehydrated and coverslipped using a permanent mounting solution (Entellan®). Positive and negative controls were included in every set of reactions for each antibody used. The expression for each cadherin was evaluated according to staining intensity [+: weak (1), ++: moderate (2), +++: strong (3)], staining area [<25% (1), 25-50% (2), >50% (3)] and membrane staining [positive (2) or negative (1)]. All the analyses were blinded for clinical and demographical details. An algorithm was then used with the sum of intensity, extension and membrane positivity values, as defined in **Supplementary Table I.** Whenever the cadherin expression score was > 3, it was considered positive. This scoring system was applied for all cadherins. In tumors comprising various paraffin blocks, a tumor was codified as positive for a given cadherin if positivity was observed at least in one block. In addition to consider isolated positivity for E-, P- and N-cadherin, we further classified the tumors according to the expression of the 3 cadherins in 8 subgroups: 1) isolated N-cadherin expression; 2) isolated E-cadherin expression; 3) isolated P-cadherin expression; 4) E- and N-cadherin co-expression; 5) P- and N-cadherin co-expression; 6) E- and P-cadherin co-expression; 7) positivity for the 3 cadherins (triple-positive); and 8) negativity for the 3 cadherins (triple-negative).

2.4. Data mining from publicly available datasets

RNA expression from a total of 154 glioblastoma multiform patients (TCGA-GBM) were downloaded from genomic data commons along with the available clinical information on 3rd April 2023. Expression data in FPKMs (fragments per kilobase of exon per million mapped fragments) for CDH1, CDH2 and CDH3 were extracted from the RNA counts file provided by

TCGA. Additional clinical information (MGMT status, Methylation class, G-CIMP methylation, IDH1 status, expression subclass and therapy class) were extracted from the original paper (47). All the analyses performed in this report were carried in R version 4.1.2. Expression was classified into high and low categories using the median value of expression as the threshold.

2.5. Statistics

The chi-square test was used to assess group differences, as appropriate. Overall survival from GBM diagnosis to patient death or last follow-up was estimated, using the Kaplan-Meier limit method. The log-rank test was used to assess group differences. A 2-tailed significance level (p-value) of 0.05 was applied. Graph Pad Prism version 9.0c software (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis and graphical data presentation.

3. Results

3.1. Clinical and neuropathological characteristics of glioblastoma series

Patient characteristics, including clinical baseline parameters and treatment decisions, are detailed in **Supplementary Table II**. The series of 313 GBM patients include a similar number of male (n=178; 56.9%) and female (n=135; 43.1%) individuals, with a mean age at diagnosis of 61,743 ± 10,483 years old. Regarding presentation at diagnosis, the majority of patients had focal deficits (n=116; 37.1%), followed by headaches (n=74; 23.6%), behaviour changes (n=65; 20.8%), and seizures (n=54; 17.3%). The mean for the initial functional status, measured by the Karnofsky Performance Scale (KPS), was 79.33 ± 10.585. Concerning tumor MRI characteristics, we could describe that the mean major axis of the tumors was 7.27 ± 2.09 cm.

The majority were unilobar (n=242; 77.3%), mostly located in the frontal (36.1%) and temporal lobes (30.4%), without ependymal (n=224; 71.6%) or corpus callosum extension (n=221; 70.6%). Less than a third of the tumors (n=76, 24.3%) were multifocal and 49 (15.7%) presented cysts. The majority of patients underwent surgical resection (n=177, 56.5%), followed by chemotherapy (temozolomide) and radiotherapy, as proposed by Stupp *et al*. (64.9%) [48]. We documented tumor recurrence or regrowth in 297 patients. Of these, 38 patients underwent surgical resection and 159 patients underwent 2nd line treatments.

Neuropathological features analyzed are summarized in **Table I.** Necrosis and vascular changes were ubiquitous in primary tumors. We identified the presence of inflammation in about a third of the cases (n=80; 25.6%). A glial component was present in up to 99.7% of the tumors (n=312), in particular gemistocytes were identified in 15.7% (n=49) and an oligodendroglial component in 11.7% (n=36). A mesenchymal component was identified in 20.1% of the tumors (n=63), more commonly than the presence of an epithelial component, which was only found in 13.1% (n=41). Based on the outlined criteria, tumors were divided in predominantly glial (n=291; 93%), predominantly mesenchymal (n=15; 4.8%), or predominantly epithelial (n=7; 2.2%).

3.2. Cadherins expression associate with clinical, imaging and neuropathological GBM features

We have studied the expression of the classical cadherins, E-, N- and P-cadherin, in the series of 313 GBM **(Figure 1A)**. In cases where preserved normal brain parenchyma was present, we observed N-cadherin expression in neurons and ganglion cells, as expected; however, no staining was found in the brain parenchyma for neither E- or P-cadherin **(Figure 1A)**.

N-cadherin was the most commonly expressed cadherin in up to 81.5% of the tumor samples (n=255) **(Table I)**. The majority of these samples had strong N-cadherin staining, since compound scores were higher than 5 in 49.9% of the cases (n=156). Moreover, membrane

staining was observed in 64.5% (n=202). In the available matched recurrence cases, virtually all tumors were N-cadherin positive (n=37; 97.4%), where a strong staining was observed in 77% of the cases (n=30) **(Table I, Figure 1C)**.

Table I. Cadherins expression in GBM and association with neuropathological features.

	GBM series	N-cadherin Positive		E-cadherin Positive		P-cadherin Positive	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value
	313 (100%)	255 (81.5%)		97 (31%)		65 (20.8%)	
Papillary structures	17 (5.4%)	15 (5.9%)	0.46	17 (17.5%)	**<0.001**	6 (9.2%)	0.126
Whorls	8 (2.6%)	5 (2%)	0.162	4 (4.1%)	0.239	6 (9.2%)	<0.001
Glomeruloid vessels	47 (15%)	39 (15.3%)	0.773	20 (20.6%)	0.063	9 (13.8%)	0.767
Myxoid stroma	18 (5.7%)	16 (6.3%)	0.404	9 (9.3%)	0.072	4 (6.2%)	0.875
Inflammation	80 (25.6%)	69 (27%)	0.502	39 (40.2%)	**0.001**	33 (50.8%)	**<0.001**
Calcifications	10 (3.2%)	10 (3.9%)	0.125	3 (3.1%)	0.945	0	0.1
Oligodend. component	36 (11.7%)	32 (12.5%)	0.643	9 (9.3%)	0.299	0	**0.014**
Gemistocytes	49 (15.7%)	49 (19.5%)	**<0.001**	13 (13.4%)	0.424	5 (7.8%)	0.058
Glial component	312 (99.7%)	254 (99.6%)	0.633	96 (99%)	0.135	65 (100%)	0.608
Mesenchymal component	63 (20.1%)	44 (17.3%)	0.75	25 (25.8%)	**0.005**	43 (66.2%)	**<0.001**
Epithelial component	41 (13.1%)	36 (14.1%)	0.263	39 (40.2%)	**<0.001**	15 (23%)	**0.007**
Predominant component							
Glial	291 (93%)	242 (94.9%)	0.981	86 (88.7%)	**<0.001**	53 (81.5%)	**0.001**
Mesenchymal	15 (4.8%)	8 (3.1%)	0.561	4 (4.1%)	0.376	9 (13.8%)	**<0.001**
Epithelial	7 (2.2%)	5 (2%)	0.489	7 (7.2%)	**<0.001**	3 (4.6%)	0.145
Tumor recurrence	38	37 (97.4%)		8 (20.5%)		25 (65.8%)	

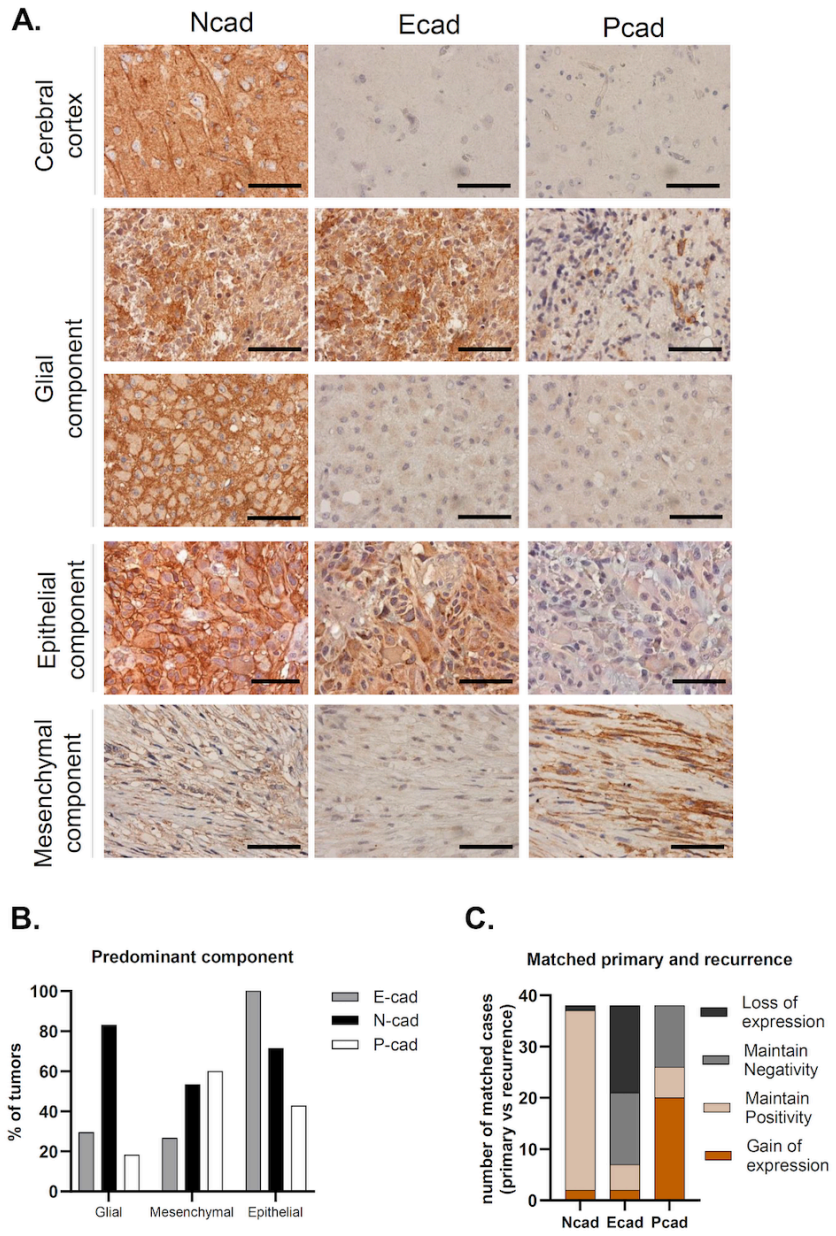

Figure 1. N-, E- and P-Cadherin expression in Glioblastoma. (A) Representative cases of predominant glial, epithelial and mesenchymal component tumors. In tumor with a predominant glial component, N-cadherin positivity was observed in the majority of the cases. In some cases, E-cadherin positivity was also observed. All tumors with a predominant epithelial component stained for E-cadherin, which was frequently accompanied by N-cadherin staining as observed in this case. Finally, tumors with a predominant mesenchymal component most commonly stained for both N-cadherin and P-cadherin (Scale bar corresponds to 50 µm). **(B)** N-cadherin expression is high in all tumor subtypes. In tumors with a predominant mesenchymal component, P-cadherin and N-cadherin expression is observed in the vast majority of cases. Strikingly, all cases with a predominant epithelial component are positive for E-cadherin. **(C)** Upon tumor recurrence, 97,4% of the tumors are positive for N-cadherin (maintain positive + gain of positivity). Gain of P-cadherin expression was the most common event which was observed in over half of the cases (51,3%). This leads to an important increase in P-cadherin positivity in recurrence when compared to the primary tumor (20,8% vs 65,8%). On the contrary, E-cadherin positivity is lower in recurrence (31% vs 20,8%), due to E-cadherin loss of staining in matched primary-recurrent cases (43,6%).

Interestingly, N-cadherin was ubiquitously expressed among tumors with different neuropathological components: glial (254 in 312 cases, 81%), mesenchymal (44 in 63 cases, 70%) and epithelial (36 in 41 cases, 88%) **(Table I, Figure 1B)**. Importantly, N-cadherin expression did not associate significantly with any particular patient or tumor characteristic.

Positivity for E-cadherin was only found in 31% (n=97) of the cases **(Table I)**. In particular, E-cadherin staining was more frequent in the cytoplasm of larger cells, with an abundant cytoplasm and defined cell borders, with occasional membrane staining (10.3%) **(Figure 1A)**. Only 9.4% of the cases had compound scores superior to 5 (n=31). Upon tumor recurrence, contrary to what was observed for N-cadherin, E-cadherin staining loss was observed in a large number of re-operated patients with initial positivity for E-cadherin (43.6%). E-cadherin positivity dropped to 20.5% (n=8) in the recurrent cases **(Table I, Figure 1C)**. Interestingly, E-cadherin positive tumors were more frequently observed in younger patients (46.7% positivity in patients <43 years old, p=0.079,), characteristically located in the temporal lobe (48%, p=0.007), with the presence of cysts (p=0.028) and a tendency for cortical involvement (p=0.094). The neuropathological characteristics that significantly correlated with immunostaining for E-cadherin included the presence of papillary structures (p<0.001) and the presence of inflammatory infiltrates (p=0.001). Strikingly, E-cadherin was positive in 95.1% (39 in 41 cases) of the cases with an epithelial component **(Table I)**. In cases with a predominant epithelial component, E-cadherin was consistently expressed in all cases (7 in 7 cases) (p<0.001). On the other hand, E-cadherin was positive in 26.7% (4 in 15 cases) of the cases with predominant mesenchymal differentiation and in 29,5% (86 in 291 cases) of the cases with predominant glial differentiation **(Table I, Figure 1B)**.

Immunostaining for P-cadherin was only found in 20.8% of primary GBM (n=65) **(Table I)**. As for E-cadherin, P-cadherin was also found in the cytoplasm of large cells, with low membrane staining (6.1% of the cases) **(Figure 1A)**. In addition, it commonly appeared in tumors containing fusiform cells **(Figure 1A)**. Strikingly, P-cadherin positivity rate in recurrence was significantly higher compared to the primary tumors (65.8% vs 20.8%) **(Table I, Figure 1C)**.

Comparing matched primary and recurrent tumors, P-cadherin gain was the most frequent event, present in 51.3% of the tumor matched pairs. The presence of a mesenchymal component also significantly correlated with positive staining for P-cadherin ($p<0.001$), which was positive in up to 66% of these cases. It was also positive in 23% of the cases with an epithelial component **(Table I, Figure 1B)**. Indeed, positivity for P-cadherin was found in 60% of the tumors where the mesenchymal component was predominant (9 in 15 cases), in 42.9% where there was a predominance of the epithelial component (3 in 7 cases), and in 18.2% of predominant glial tumors (53 in 291 cases) ($p<0.001$). In addition, we identified the presence of lymphocytic infiltrates in about 50% of P-cadherin positive cases ($p<0.001$).

3.3. Cadherins co-expression profiles improve the identification of GBM subgroups

We further classified tumors into 8 subgroups according to the co-expression of the three studied cadherins. The expression of more than one cadherin was identified in 124 tumors (39.6%), while only 22 tumors expressed all the three cadherins (triple positive tumors, 7%). Moreover, the immunostaining was negative for N-, E- and P-cadherin in 40 tumors (12.8%, triple negative tumors). Most commonly, tumors expressed only N-cadherin (n=137, 43.8%) or co-expressed E- and N-cadherin (n=67, 21.4%), as shown in **Table II** and **Figure 2A**. Interestingly, upon tumor recurrence, the most common profiles were the co-expression of P- and N-cadherin (46.2%) or the isolated expression of N-cadherin (30.8%) **(Figure 2A)**. Cadherins expression profiles changed in 69.2% of the cases. Curiously, all E-cadherin positive cases, upon tumor recurrence, showed concomitant expression of P- and N-cadherin **(Figure 2A)**.

In tumors with more than one FFPE block, we evaluated the heterogeneity of each cadherin subgroup. Over 50% of the tumors showed intratumoral cadherin expression heterogeneity, including 4 distinct subgroups in 1.7% of the tumors. Curiously, N-cadherin expression was heterogeneous in 40% of the tumors and E-cadherin in 28% of the tumors, while P-cadherin was heterogeneous in only 16% of the tumors.

When considering the combined expression of the three cadherins, we further evaluated putative significant correlations with clinical, imaging and neuropathological characteristics. Clinical presentation associated to cadherin co-expression profiles **(Table II).** In particular, patients with tumors that expressed epithelial cadherins more frequently presented focal deficits (P-cadherin, p=0.024 and co-expression of E and P-cadherin, p=0.018); while headache was the most common presentation in patients with tumors that showed isolated E-cadherin expression (p=0.015).

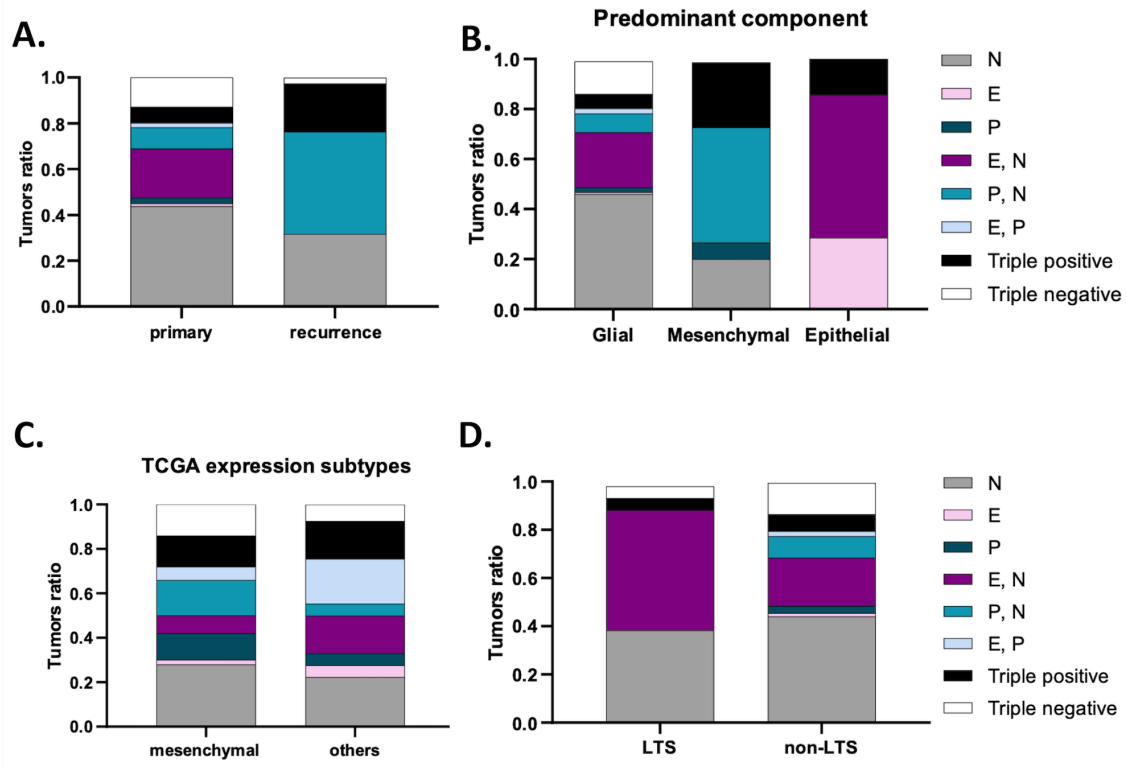

Figure 2. Cadherin expression subgroups in Glioblastoma. (A) Compared to primary tumors, the most frequent subgroups in recurrent tumor are P- and N-cadherin co-expression (46,2%) and N-cadherin expression (30,8%). **(B)** Tumors with a predominant epithelial component expressed E-cadherin, E- N- cadherin or triple positive subgroups. For mesenchymal tumor, the subgroups expressed were P- N- cadherin, triple positive, P-cadherin and N- cadherin. **(C)** Analysis of data from the TCGA, confirmed a main expression of P-N cadherin, N-cadherin, triple positive and N-cadherin subgroups in the mesenchymal transcriptional subtype. P-N- co-expression significantly correlated with the mesenchymal subtype (p=0,033). **(D)** Tumors from long-term survival patients exhibited more frequently E-N-cadherin co-expression, N-cadherin, triple positive or triple negative subgroups (p=0,013).

These expression profiles also correlated with age, specifically the isolated expression of E-cadherin was more common in younger patients (p=0.015). Tumor location was more common in the frontal lobe for isolated N-cadherin expression tumors (p=0.024) and the temporal lobe for the co-expression of E- and N-cadherin (p=0.022). Other imaging features correlated with cadherin expression subgroups included: cyst formation (co-expression E and N-cadherin, p<0.001; and a tendency for E-cadherin, p=0.057), multifocality (P-cadherin expression, p=0.011; and co-expression P- and N-cadherin, p=0.024), ependymal extension (P-cadherin expression, p=0.030; and E- and P-cadherin co-expression, p=0.036), and corpus callosum extension (tendency for P- and N- cadherin co-expression, p=0.055).

When evaluating for distinct neuropathological phenotypes, we observed that tumors with a predominant sarcomatous/mesenchymal differentiation predominantly exhibited N- and P-cadherin co-expression (44.4%), or were positive for the 3 cadherins (33.3%), or expressed only P-cadherin (11.1%) or only N-cadherin (11.1%) (p<0.001) **(Figure 2B)**. Predominant epithelial differentiation was observed in tumors showing only E-cadherin expression (28.6%), E- and N-cadherin co-expression (28.6%) and the expression of the 3 cadherins (triple positive, 42.9%) (p<0.001) **(Figure 2B)**. Tumors with predominant glial expression had most frequently only N-cadherin expression (46.1%), E- and N-cadherin co-expression (21.5%) or were negative for the 3 cadherins (13.5%) **(Figure 2B)**.

Based on phenotypic differentiation results, we further explored cadherin expression differences according to transcription subtypes, particularly the mesenchymal subtype, using the online available TCGA data. Indeed, this data showed that tumors with mesenchymal transcription subtype were enriched for isolated N-cadherin expression (28%), P- and N-cadherin co-expression (16%), triple positivity (14%), triple negativity (14%) and isolated P-cadherin expression (12%) subgroups. When compared to tumors with other subtypes, P- and N-cadherin co-expression specifically associated with the presence of a mesenchymal subtype (p=0.019) **(Figure 2C)**.

Table II. Cadherins co-expression profiles in glioblastomas and association with clinical, imaging and neuropathological characteristics.

	GBM series	Ncad		Ecad		Pcad		Ecad/ Ncad		Pcad/ Ncad		Ecad/ Pcad		Triple positive		Triple negative	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value
	313 (100%)	137 (43.8%)		4 (1.3%)		8 (2.6%)		67 (21.4%)		29 (9.3%)		6 (1.9%)		22 (7%)		40 (12.8%)	
Presentation																	
Focal deficits	116 (37.1%)	52 (38%)	0.773			6 (75%)	**0.024**	17 (25.4%)	**0.025**	12 (41.4%)	0.615	5 (83.3%)	**0.018**	8 (36.4%)	0.944	16 (40%)	0.681
Headache	74 (23.6%)	24 (17.5%)	**0.024**	3 (75%)	**0.015**			19 (28.4%)	0.307	10 (34.5%)	0.15			6 (27.3%)	0.679	12 (30%)	0.312
Behaviour	65 (20.8%)	35 (25.5%)	0.066	1 (25%)	0.834	2 (25%)	0.766	13 (19.4%)	0.757	4 (13.8%)	0.333	1 (16.7%)	0.803	4 (18.2%)	0.757	5 (12.5%)	0.169
Seizures	54 (17.3%)	23 (16.8%)	0.849					18 (26.9%)	**0.019**	3 (10.3%)	0.303			3 (13.6%)	0.643	7 (17.5%)	0.965
Finding	4 (1.3%)	3 (2.2%)	0.206											1 (4.5%)	0.158		
Age (mean)	61,743 ± 1,483	62,1± 11,0	0.848	49 ± 16,39	**0.015**	66,25 ± 6,409	0.299	60 ± 11,6	0.264	61,103 ± 8.491	0.55	68,833 ± 5,037	0.139	62,2 ± 10	0.97	65,4 ± 11,6	0.055
Location																	
Frontal	113 (36.1%)	59 (43.1%)	**0.024**	1 (25%)	0.643	3 (37.5%)	0.934	23 (34.3%)	0.734	9 (31%)	0.552	1 (16.7%)	0.367	5 (22.7%)	0.177	12 (30%)	0.391
Temporal	95 (30.4%)	29 (21.2%)	**0.002**	1 (25%)	0.815	3 (37.5%)	0.657	28 (41.8%)	**0.022**	11 (37.9%)	0.353	1 (16.7%)	0.463	10 (45.5%)	0.111	12 (30%)	0.959
Parietal	70 (22.4%)	33 (24.1%)	0.52	1 (25%)	0.899	1 (12.5%)	0.499	11 (16.4%)	0.189	5 (17.2%)	0.489	4 (66.7%)	**0.008**	5 (22.7%)	0.966	10 (25%)	0.67
Deep	15 (4.8%)	6 (4.4%)	0.764	1 (25%)	0.057	1 (12.5%)	0.303	2 (3%)	0.436	1 (3.4%)	0.723			1 (4.5%)	0.955	3 (7.5%)	0.392
Other	20 (6.4%)	10 (7.3%)	0.563					3 (4.5%)	0.472	3 (10.3%)	0.362			1 (4.5%)	0.715	3 (7.5%)	0.759
Cysts	49 (15.7%)	33 (24.1%)	0.65	2 (50%)	0.057	0	0.218	22 (32.8%)	**<0.001**	1 (3.4%)	0.057	2 (33.3%)	0.229	0	**0.036**	2 (5%)	**0.047**
Multifocal	76 (24.3%)	35 (25.5%)	0.645	1 (25%)	0.973	5 (62.5%)	**0.011**	10 (14.9%)	0.044	12 (41.4%)	**0.024**	2 (33.3%)	0.602	6 (27.3%)	0.734	5 (12.5%)	0.063
Corpus callosum ext.	92 (29.4%)	40 (29.2%)	0.946	1 (25%)	0.846	4 (50%)	0.195	17 (25.4%)	0.415	13 (44.8%)	0.055	2 (33.3%)	0.831	7 (31.8%)	0.796	8 (20%)	0.163
Ependymal extension	89 (28.4%)	39 (28.5%)	0.991	2 (50%)	0.336	5 (62.5%)	**0.03**	14 (20.9%)	0.123	9 (31%)	0.745	4 (66.6%)	**0.036**	7 (31.8%)	0.715	9 (22.5%)	0.373
Predominant component																	
Glial	291 (93%)	135 (97.8%)	**0.003**	2 (50%)	**<0.001**	7 (87.5%)	0.541	63 (94%)	0.703	22 (75.9%)	**<0.001**	6 (100%)	0.498	17 (77.3%)	**0.003**	40 (100%)	0.063
Mesenchymal	15 (4.8%)	3 (2.2%)	0.057			1 (12.5%)	0.303			7 (24.1%)	**<0.001**			4 (18.2%)	**0.002**		
Epithelial	7 (2.2%)			2 (50%)	**<0.001**			4 (6%)	**0.020**					1 (4.5%)	0.449		
Treatment																	
No	96 (30.7%)	46 (33.6%)	0.327			3 (37.5%)	0.673	13 (19.4%)	0.086	11 (37.9%)	0.375	1 (16.7%)	0.454	7 (31.8%)	0.904	15 (37.5%)	0.317
RT	19 (6.1%)	7 (5.1%)	0.531	1 (25%)	0.111			3 (4.5%)	0.540	1 (3.4%)	0.536	1 (16.7%)	0.274			6 (15%)	**0.011**
QT+RT	8 (2.5%)	3 (2.2%)	0.718					2 (3%)	0.803	1 (3.4%)	0.750					2 (5%)	0.296
Stupp	189 (60.4%)	81 (59.1%)	0.615	3 (75%)	0.557	5 (62.5%)	0.916	49 (73.1%)	0.079	16 (55.2%)	0.524	4 (66.7%)	0.764	15 (68.2%)	0.458	17 (42.5%)	0.096

3.4. Cadherins expression profiles define GBM patients' prognosis

As expected, patients' age (HR=1.405; p=0.004), functional status (KPS 50; HR=1.772; p=0.006), surgical decision (biopsy; HR=2.329; p<0.001) and complimentary treatment (no treatment; HR=4.73; p<0.001) were validated as prognostic factors in this particular GBM series. Thus, survival analysis was used to correlate cadherins expression with GBM patients' prognosis.

In particular, N-cadherin expression identified tumors with improved progression-free survival (median 6 months, log rank test, p=0.033) and overall survival (median 12 months, log rank test, p=0.001) **(Figure 3A)**, while a significant decrease in progression-free survival and overall survival was observed in tumors positive for P-cadherin expression (log rank test, median 3 months and 7 months, p=0.028 and p=0.024, respectively) **(Figure 3C).** E-cadherin staining identified tumors with a longer time to recurrence (7 months, log rank test, p=0.008) and a better overall survival (14 months, log rank test, p=0.039) **(Figure 3B)**.

Interestingly, the combined expression of cadherins also stratified patients' prognosis, both progression-free and overall survival **(Figure 3D and Table III)**. For instance, Kaplan-Meier survival curves showed a survival benefit for E- and N-cadherin co-expression for both progression free (log rank test, p=0.005, median 7 months) and overall survival (log rank test, 14 months, p=0.005) **(Figure 3D)**. On the contrary, P- and N-cadherin or E- and P-cadherin co-expression showed the shorter times to recurrence (median 2 and 3 months, log rank test, p=0.046 and p=0.093, respectively), while E- and P-cadherin co-expression and single P-cadherin expression showed a tendency for worse overall survival (log rank test, 9 months, p=0.078 and 9 months, p=0.055, respectively) **(Figure 3D)**.

Univariate analysis identified three subgroups with worse progression free survival: E- and P-cadherin co-expression (HR=2.203, p=0.035), P- and N-cadherin co-expression (HR=1.91, p=0.008) and triple negative tumors (HR=1.712, p=0.021); and four subgroups with worse overall survival: E- and P-cadherin co-expression (HR=2.728, p=0.020), P-cadherin (HR=2.611,

p=0.012), triple negative tumors (HR=1.794, p=0.005) and P- and N- co-expression (HR=1.619, p=0.036) **(Table III)**.

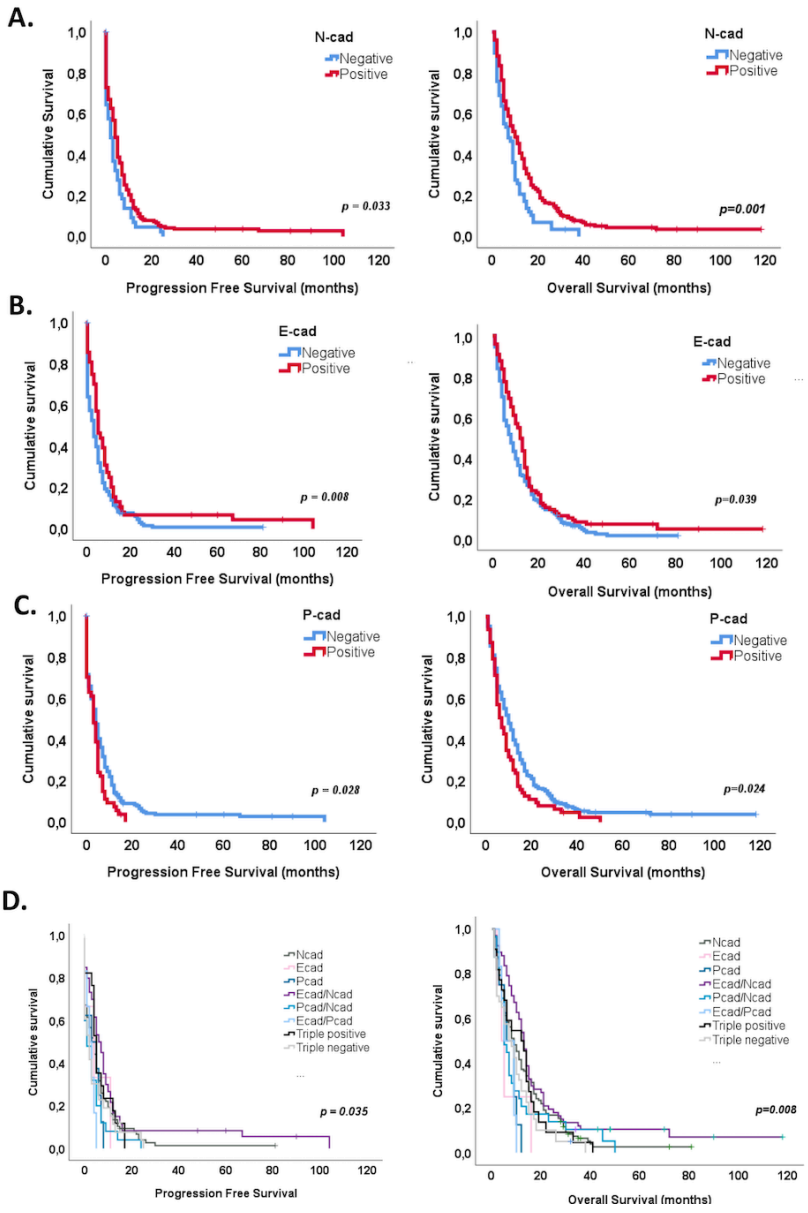

Figure 3. Survival analysis according to Cadherin expression. (A) N-cadherin expression in GBM showed a significant improve in both progression-free (p=0.033) and overall survival (p=0.001). **(B)** A significant survival benefit was also observed in tumors with E-cadherin expression (p=0.008 for progression-free survival and p=0,039 for overall survival. **(C)** On the contrary, P-cadherin expression significantly marked tumors with a shorter time for recurrence (p=0,028) and survival (p=0,024). **(D)** Kaplan-Meier curves identified a survival benefit for E-N- cadherin co-expression subgroup (p=0,005 for both PFS and OS). P-N-cadherin co-expression (p=0,046) subgroup significantly associated to a worse progression-free survival, while E- P- cadherin co-expression (p=0,078) and P-cadherin (p=0,055) subgroups showed a tendency for worse overall survival.

Table III. Univariate analysis of Cadherins expression profiles and association with prognosis.

		Progression Free Survival				Overall Survival			
		HR	95.0% CI		p-value	HR	95.0% CI		p-value
			Inferior	Superior			Inferior	Superior	
Surgery	**Surgery**	1				1			
	Biopsy	2.120	1.626	2.763	**<0.001**	2.329	1.842	2.945	**<0.001**
Treatment	**Yes**	1				1			
	No	13.244	8.492	20.665	**<0.001**	4.730	3.547	6.308	**<0.001**
KPS	**100**	1				1			
	50	0.505	0.056	4.550	0.543	1.772	1.183	2.654	**0.006**
	60	0.580	0.078	4.318	0.595	1.411	0.866	2.298	0.167
	70	0.587	0.080	4.295	0.600	2.575	1.223	5.421	**0.013**
	80	0.430	0.059	3.105	0.403	2.596	1.035	6.508	**0.042**
	90	0.391	0.054	2.826	0.352	1.599	1.022	2.503	**0.040**
Age	**< 65 yo**	1				1			
	> 65 yo	1.258	0.974	1.625	0.079	1.400	1.113	1.762	**0.004**
Ncad	**Negative**	1				1			
	Positive	0.725	0.522	1.007	0.055	0.627	0.468	0.840	**0.002**
Ecad	**Negative**	1				1			
	Positive	0.716	0.546	0.938	**0.016**	0.779	0.608	0.998	**0.048**
Pcad	**Negative**	1				1			
	Positive	1.367	1.003	1.863	**0.048**	1.364	1.029	1.806	**0.031**
Cadherin co-expression	**Ecad/ Ncad**	1				1			
	Ncad	1.367	0.984	1.900	0.063	1.316	0.969	1.787	0.079
	Ecad	1.507	0.471	4.826	0.490	2.550	0.923	7.041	0.071
	Pcad	1.864	0.884	3.933	0.102	2.611	1.239	5.502	**0.012**
	Pcad/ Ncad	1.910	1.186	3.076	**0.008**	1.619	1.033	2.536	**0.036**
	Ecad/ Pcad	2.203	1.068	5.868	**0.035**	2.728	1.169	6.366	**0.020**
	Triple positive	1.194	0.683	2.087	0.533	1.427	0.875	2.328	0.154
	Triple negative	1.712	1.084	2.704	**0.021**	1.794	1.196	2.690	**0.005**

Multivariate analysis identified E- and P-cadherin co-expression (HR=2.96, p=0.020) as a significant independent factor for worse progression-free survival **(Table IV)**. E-cadherin (HR=4.24, p=0.017), E- and P-cadherin co-expression (HR=3.35, p=0.021), P-cadherin

(HR=2.33, p=0.036), and triple positivity (EPN) (HR=2.11, p=0,011), were also found as significant independent factors for worse overall survival **(Table IV)**. In concordance with these results, of the 21 long-term survivors (LTS) (6.7%) with in our series, 42.9% were N- and E-cadherin co-expressing tumors, compared to the 19.9% expression in non-LTS (p=0.013); 38.1% of the GBM showed N-cadherin expression, 9.5% were triple positive and other 9.5% were triple negative tumors. Strikingly, the tumors from LTS patients did not show isolated expression of epithelial cadherins (E- and P-cadherins) **(Figure 2D)**.

Table IV. Multivariate analysis of Cadherins expression profile and association with prognosis.

		Progression Free Survival				Overall Survival			
		HR	95.0% CI		p-value	HR	95.0% CI		p-value
			Inferior	Superior			Inferior	Superior	
Mesenchymal Component	**yes**	1.000				1.000			
	no	1.060	0.670	1.660	0.811	0.810	0.530	1.250	0.345
Epithelial Component	**yes**	1.000				1.000			
	no	0.930	0.580	1.470	0.743	0.720	0.450	1.140	0.160
Cadherins co-expression	**Ecad/N-cad**	1.000				1.000			
	Ncad	0.990	0.690	1.440	0.979	1.300	0.900	1.870	0.162
	Ecad	2.200	0.650	7.430	0.205	4.240	1.290	13.930	**0.017**
	Pcad	1.330	0.600	2.980	0.482	2.330	1.050	5.130	**0.036**
	Pcad/ Ncad	1.340	0.770	2.360	0.303	1.440	0.850	2.460	0.178
	Ecad/ Pcad	2.960	1.180	7.400	**0.020**	3.350	1.330	8.440	**0.010**
	Triple pos.	1.190	0.640	2.210	0.577	2.110	1.190	3.760	**0.011**
	Triple neg.	1.090	0.660	1.820	0.728	1.490	0.910	2.450	0.113
Surgery	**Surgery**	1.000				1.000			
	Biopsy	1.760	1.290	2.390	**<0.001**	1.550	1.170	2.060	**0.003**
Treatment	**yes**	1.000				1.000			
	no	15.000	9.020	24.930	**<0.001**	4.570	3.300	6.330	**<0.001**
KPS	**100**	1.000				1.000			
	50	0.150	0.020	1.390	0.094	0.700	0.080	6.170	0.752
	60	0.250	0.030	1.900	0.179	2.400	0.320	18.040	0.396
	70	0.240	0.030	1.790	0.162	2.190	0.300	16.220	0.442
	80	0.210	0.030	1.550	0.125	1.520	0.210	11.150	0.681
	90	0.200	0.030	1.510	0.118	1.310	0.180	9.660	0.790
Age	**< 65 yo**	1.000				1.000			
	> 65 yo	1.210	0.920	1.600	0.176	1.270	0.980	1.660	0.072

4. Discussion

E-cadherin, P-cadherin and N-cadherin expression function and impact in prognosis is well established in various systemic cancers. It is nevertheless important to remember that this consensus is accepted for epithelial tumors and, although E- to N-cadherin switch is frequently used as a marker of EMT in cancers of other origins, the factual involvement of these proteins in processes akin to the EMT in non-epithelial tumors remains to be adequately studied and clarified. Actually, the EMT process became recently accepted as a continuum between these two definable states, often achieving hybrid phenotypes, with increased cell plasticity. In this context, cadherin expression regulation will most likely follow the cell-cell adhesion requirements of the different EMT states.

Given the knowledge gap in cadherin expression in glial tumors, our main goal was to characterize the expression of the most studied classical cadherins (E-, N- and P-cadherins) in a large and representative series of GBM. To do so, and taking into account GBM intratumoral heterogeneity, we marked the expression of these proteins in all available paraffin blocks of 313 patients, in a total of 727 samples. Our main findings were the following: 1) N-cadherin is the most frequent cadherin expressed in GBM, present in over 80% of the cases, followed by E-cadherin in about a third of the tumors and P-cadherin in 20%; 2) expression of epithelial cadherins (E- and P-cadherin) determines a distinct imaging and neuropathological profile in GBM; and 3) expression of epithelial cadherins have a significant detrimental impact in GBM patient's prognosis. In addition to showing distinct associations with clinical and neuropathological characteristics, it is notable the distinct behavior of E- and P-cadherins expression upon tumor recurrence. There was a clear P-cadherin dominance and a loss of E-cadherin expression in recurrent lesions compared with primary tumors. Survival analysis further supported P-cadherin as a marker of tumor aggressiveness, as P-cadherin expressing tumors were significantly correlated with a worse patient prognosis. On the

contrary, E-cadherin expression was observed in patients with a significant increase in progression-free and overall survival.

These results, although corroborated by robust morphological correlations, only considered the expression of each isolated cadherin and may thus represent a simplified version of an intricately regulated cellular mechanism between them. In fact, the mechanistic reciprocities between the expression of distinct cadherins have been already suggested, although poorly explored in tumor pathogenesis. Examples include the co-expression of R- and E-cadherin that suppress the progression and metastasis in salivary adenoid cystic carcinomas [49], the combined expression of cadherin-6 and cadherin-11 associated with lymph node metastasis and poor prognosis in oral squamous cell carcinoma [50], and the co-expression of E- and P-cadherin which associated to poor prognosis in breast cancer [23]. In particular, further studies of the mechanisms involving E- and P- co-expression led to the proposal of P-cadherin as a marker of an intermediate EMT phenotype [51].

Hence, we expanded our analysis and evaluated tumor characteristics considering the simultaneous expression of the three classical cadherins. This allowed us to not only identify distinct tumor subgroups, as, more importantly, to confirm the importance of the cadherin interplay in the biological outcome of GBM. For example, the isolated expression of N-cadherin was able to identify the most common GBM profiles (up to 43.8% of the cases), which are the ones commonly found in the frontal lobe, with a typical glial component and few inflammatory infiltrates. Importantly, when N-cadherin expression is combined with E- or P-cadherin, divergent tumor subtypes are observed: E- and N-cadherin co-expressing tumors are frequently located in the temporal lobe, with cortical involvement and cysts, and frequently exhibit an epithelial component; while GBM positive for P- and N-cadherin frequently displays a mesenchymal component, with frequent immune infiltrates. Finally, survival analysis highlights tumors with E- and N-cadherin co-expression as a subgroup with significantly improved progression-free and overall survival, while P- and N-cadherin subgroup had shorter times to recurrence and worse overall survival. It is also noteworthy

that, in our data, the P-cadherin increase in recurrence is always accompanied by N-cadherin expression, while co-expression of E-cadherin and N-cadherin was not observed in any recurrent case. Tumors without N-cadherin expression, but with the expression of epithelial cadherins, commonly exhibit a distinctive neuropathological and MRI patterns, and collectively are subgroups with a worse prognosis.

Taken together, these results suggest that although N-cadherin is the dominant cadherin in GBM, its function can be strongly modulated by the expression of epithelial cadherins. When associated with E-cadherin, it frequently leads to an epithelial-differentiated tumor with a distinct clinical profile and a survival benefit. However, when combined to P-cadherin expression, it leads to tumors with a predominant mesenchymal differentiation, with reduced time for recurrence and survival. In addition, this is the most frequent subgroup upon tumor recurrence. These results led us to hypothesize that the mesenchymal state in glioblastoma is most likely reflected by the combined expression of P and N-cadherins. In fact, these results were further supported by data leveraged from TCGA, showing a significant association between the mesenchymal transcriptional profile and P- and N-cadherin co-expression.

P-cadherin positive status, in our analysis, clearly marks for the worse prognosis subgroups. In particular, isolated P-cadherin or E- and P-cadherin co-expression were found as independent predictors for worse overall survival, while also displaying an imaging profile suggestive of multifocality and propensity to dissemination. This is in concordance with recent reports which associated P-cadherin expression to GBM aggressiveness, through an increase in cell invasion and migration, as well as a decrease in patient survival [46]. In addition, we curiously observe that these tumors often display both mesenchymal or epithelial differentiation, but not a homogeneous differentiation, denoting perhaps the presence of heterogeneous cancer cell populations with increased phenotypic plasticity. Thus, our results suggest that neither E-cadherin, nor N-cadherin, expression are able to counteract the disadvantageous biology brought by P-cadherin expression. An apparent profile with increased cancer cell plasticity and markers for enhanced infiltrative potential

seems to be denoted by the expression of epithelial cadherins, either P-cadherin expression or E- and P-cadherin co-expression, which we interpret in the context of the possible acquisition of a hybrid EMT state.

The role of E-cadherin, also an epithelial cadherin, with infrequent expression in GBM, is less clear. Our results show a potential dichotomy in the role of this protein. By one hand, when it was co-expressed with N-cadherin, a subgroup with distinct neuropathological, MRI features and improved survival is identified. On the other hand, tumors with isolated expression of the E-cadherin seem to portend a worse prognosis, although they exhibit a similar neuropathological and imaging profile as E- and N-cadherin expressing tumors. Published reports on epithelioid GBM describe this neuropathological and imaging profile, and typically identify tumors with an aggressive course [52, 53]. It is thus possible that, as described for other tumors, the expression of other concomitant cadherins, and hence activation of different cellular programs, and not expression of E-cadherin per se, will define tumor overall characteristics and aggressiveness.

Finally, the biology of triple positive and triple negative tumors is equivocal. Triple negative tumors do not relate to specific patient characteristics or prognosis. Triple positive tumors present all three morphological components, although significantly associate to predominance of a mesenchymal component. They also are a frequent subgroup in tumor recurrence. These results point to a closer similarity to the P- and N- subgroup. In accordance with these results, we propose a reformulation of the rigid concept of E- to N-cadherin switch as a marker of EMT activation and tumor aggressiveness in GBM. In fact, we hypothesize that tumor characteristics are more likely dictated by the interplay between the expression of different cadherins and considering the expression of a single cadherin may contribute to equivocal results. N-cadherin tumors are the most frequent in our analysis and reflect the average GBM. Our results propose P- cadherin and N-cadherin interplay as a contributor for the mesenchymal-like state in GBM. The presence of an intermediate, more plastic phenotype, with increased tumor aggressiveness, is most likely reflected by the presence of

epithelial cadherins, P-cadherin or E- and P-cadherin co-expression. Lastly, GBM is not an epithelial tumor and is therefore less obvious if an epithelial differentiation would present a protective effect. If so, then such putative protective epithelial state in GBM is clearly reflected by an E- and N-cadherin interaction. Further studies inquiring the mechanisms underlying cadherin interplay are warranted.

5. Conclusions

In this study, we document the expression of N-, E-, and P-cadherin in GBM. Our findings establish that cadherin co-expression is associated with consistent clinical, imaging, and neuropathological differences, ultimately correlating with patient prognosis. Additionally, we challenge the notion that an E- to N-cadherin switch is a definitive marker of an EMT-like program in GBM. Instead, we propose that cadherin co-expression subgroups serve as indicators of distinct differentiation stages within GBM.

6. References

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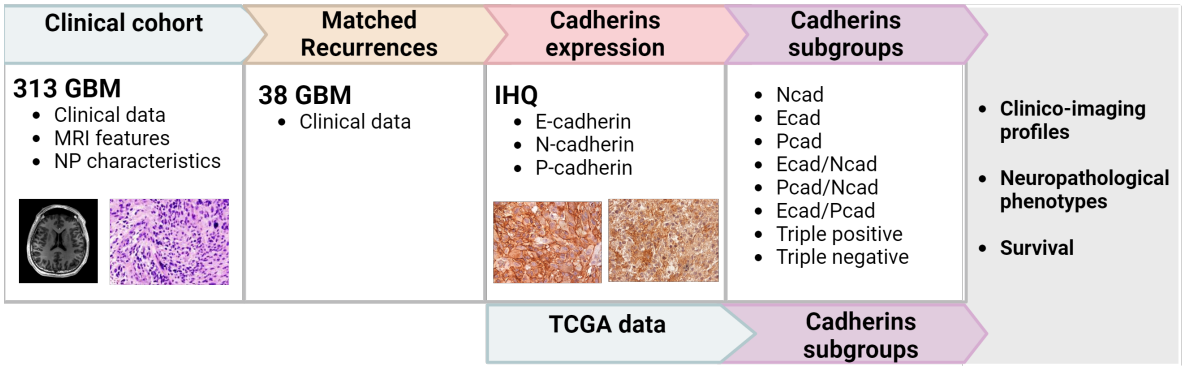
7. Supplementary data

Supplementary Figure 1. Study design



Supplementary Table I. Classification algorithm

Intensity	Extension	Membrane
+ (1 point)	<25% (1 point)	no (1 point)
++ (2 points)	25-50% (2 points)	yes (2 points)
+++ (3 points)	>50% (3 points)	

CHAPTER VI: Cadherins as biomarkers of ZEB/PD-L1 interactions in Glioblastoma

Cadherins as biomarkers of ZEB/PD-L1 interactions in Glioblastoma

1. Introduction

Glioblastoma (GBM) is an aggressive brain tumor characterized by rapid growth and resistance to treatment. Central to its malignancy are the processes of immunosuppression and immune evasion, both influenced by intricate molecular interactions [1–3]. An example of a mechanism of immune evasion is the expression of PD-L1 by the tumor, which has been extensively described [4–6] and used as a therapeutical target in GBM [2,3,7]. Recently, immune checkpoint expression and other mechanisms of immune evasion have been coupled to the EMT (epithelial-to-mesenchymal transition) cellular program [8,9], which is driven by specific transcription factors, such as ZEB1 and ZEB2, culminating in the repression of E-cadherin in epithelial cancers [9].

This regulatory network between ZEB1/ZEB2 and PD-L1 are based on transcription activation through ZEB1 direct binding to the promoter of the *PD-L1* gene, or through indirect regulation by intermediary factors, such as TGF-ß or miRNA-200 [11–13]. On the other hand, PD-L1 expression reinforces the EMT, creating a self-sustained loop that promotes tumor aggressiveness [14,15]. Furthermore, PD-L1 expression has been documented in both the intermediate and the mesenchymal states of EMT [16].

The role of ZEB1/ZEB2 in GBM is an emerging area of research, as their activation endows GBM cells with increased migratory and invasive properties, higher tumor plasticity and, ultimately, resistance to therapy [17–22]. However, the intricate regulation of EMT-like programs in GBM, and how cadherin expression reflects different EMT stages in these CNS tumors, remains poorly understood. Recently, we hypothesized that the combined expression of E-, N- and P-cadherins was a better prediction of EMT activation and status in GBM, with isolated expression of P-cadherin and co-expression of E- and P-cadherin as

markers of an intermediate EMT state, while co-expression of P- and N-cadherin would stain a more mesenchymal state[23]. In another study, we reported PD-L1 expression as a poor prognostic marker in the same series of GBM [24].

Therefore, the present study aims to confirm the existence of a cooperative mechanism between PD-L1 expression and the EMT-like program in GBM, being EMT represented by the expression of ZEB1/ZEB2. In addition, we aim to determine whether the expression of E-, N- and P-cadherins accurately reflect the convergence of these mechanisms.

2. Materials and Methods

2.1. Patients and materials

A retrospective series of patients diagnosed with glioblastoma (GBM), between 2010 and 2017, at the Neurosurgery Department, Hospital de Santo António (Porto, Portugal), has been retrieved. Formalin-fixed and paraffin-embedded (FFPE) tumor tissues were histologically analyzed for all primary tumors, as well as for matched local recurrences.

This GBM series has been previously characterized regarding its clinical data, treatment, patient follow-up and survival statistics [23,24]. Clinical data includes the evaluation of initial blood counts, particularly lymphocyte count and neutrophil-to-lymphocyte ratio [24].

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, as well as under the international Helsinki declaration. Moreover, it has also been approved by the ethics committee at Centro Hospitalar de Santo António (DEFI 104-17 (093-DEFI/091-CES).

2.2. Neuropathological evaluation

All cases were reviewed by an experienced neuropathologist, blinded to clinical and demographical details. Neuropathological features were analyzed in hematoxylin and eosin staining from the initial tumor samples. Neuropathological phenotypes were evaluated and the presence of distinct areas of differentiation were documented according to the following features: 1) the glial component, which included agglomerates of fibrillary astrocytes, gemistocytes, multinucleated giant cells or cells with oligodendroglial features; 2) the epithelioid component, which has been considered when morphologic epithelial appearance, with large and polygonal shape cells were present, with abundant cytoplasm and defined cell border; 3) a sarcomatous component was also considered, whenever areas of sarcomatous or mesenchymal differentiation were observed, often comprising chondral or osteochondral elements. The predominant component (>50% of the tumor) was documented as well.

2.4. Immunohistochemical Staining

Immunohistochemical (IHC) staining was performed with specific monoclonal antibodies for the ZEB1 and ZEB2 in 3 µm sections of a total of 164 tumor samples, including 36 matched-recurrent tumors, according to the following protocol: ZEB-1 (1:150, mouse, clone 3G6, Invitrogen, USA) and ZEB-2 (1:500, rabbit, clone PA5-82094, Invitrogen, USA). Briefly, dewaxing and epitope recovery was performed using Dewax and EDTA. The downstream IHC protocol was performed in the Lab VisionTM Autostainer 480S, using the Epredia UltraVision™ Quanto-HRP Detection System. The time for primary antibody incubation was 60 minutes. All reactions were revealed with 3,3'-diaminobenzidine (DAB) chromogen. Tissues were then counterstained with HIGHDEF® hematoxylin (Enzo, Farmingdale, USA), dehydrated and coverslipped using a permanent mounting solution (Entellan®). Positive and negative controls were included in every set of reactions for each antibody used. The expression for ZEB1 and ZEB2 was evaluated according to the intensity of their nuclear staining [+: weak (1), ++:

moderate (2), +++: strong (3)] and the area of tumor stained [<25% (1), 25-50% (2), >50% (3)]. All the analyses were blinded for clinical and demographical details. An algorithm was then used with the sum of intensity and area. Whenever the expression score was > 3, it was considered positive. In addition to considering the expression of ZEB1 and ZEB2 in separately, we created 4 subgroups considering the combined expression of both transcription factors: ZEB1+ZEB2+ (double positivity), ZEB1+ (isolated ZEB1 expression), ZEB2+ (isolated ZEB2 expression) and ZEB1-ZEB2- (negative for both).

Classical N-, E- and P-cadherins and PD-L1 have been previously evaluated for the whole cohort (n=313 patients), as previously reported [23,24].

2.5. Statistics

The chi-square test, Mann-Whitney and Kruskall-Wallis tests were used to assess group differences, as appropriate. Overall survival from GBM diagnosis to patient death or last follow-up was estimated, using the Kaplan-Meier limit method. The log-rank test was used to assess group differences. A 2-tailed significance level (p-value) of 0.05 was applied. Graph Pad Prism version 9.0c software (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis and graphical data presentation.

3. Results

3.1. ZEB1 and ZEB2 expression in primary GBM and matched recurrent cases

ZEB1 expression was observed in 75,7% (n=125) of the cases and ZEB2 in 67,2% (n=111) **(Figure 1A-E)**. High expression was observed in 29,8% (n=37) and 21% (n=29) of the tumors for ZEB1 and ZEB2, respectively.

The combined ZEB1+ZEB2+ expression was observed in 53,9% of the tumors (n=89), while there was no expression for either ZEB1 nor ZEB2 in 20 (12,12%) of the tumors **(Table I)**. In the

available matched recurrence cases, expression rates of both ZEB1 and ZEB2 significantly increased. ZEB1 was positive in 80,6% of recurrent cases (n=29), with a gain in 28,5% of matched-tumors **(Figure 1F)**. Positivity for ZEB2 was observed in 88,9% of the tumors (n=32), with a gain of expression in 57% of the matched-recurrent tumors **(Figure 1F)**.

Evaluation of neuropathological characteristics correlated the absence of ZEB1 and ZEB2 expression with tumors with a predominant epithelial component (p=0.012). Although not statistically significant, all tumors with a predominant mesenchymal component were positive for ZEB1 and up to 67% were positive for ZEB2. In addition, we observed a consistent presence of glial, epithelial and mesenchymal components in tumors exhibiting ZEB1 and ZEB2 positivity **(Supplementary Table I)**.

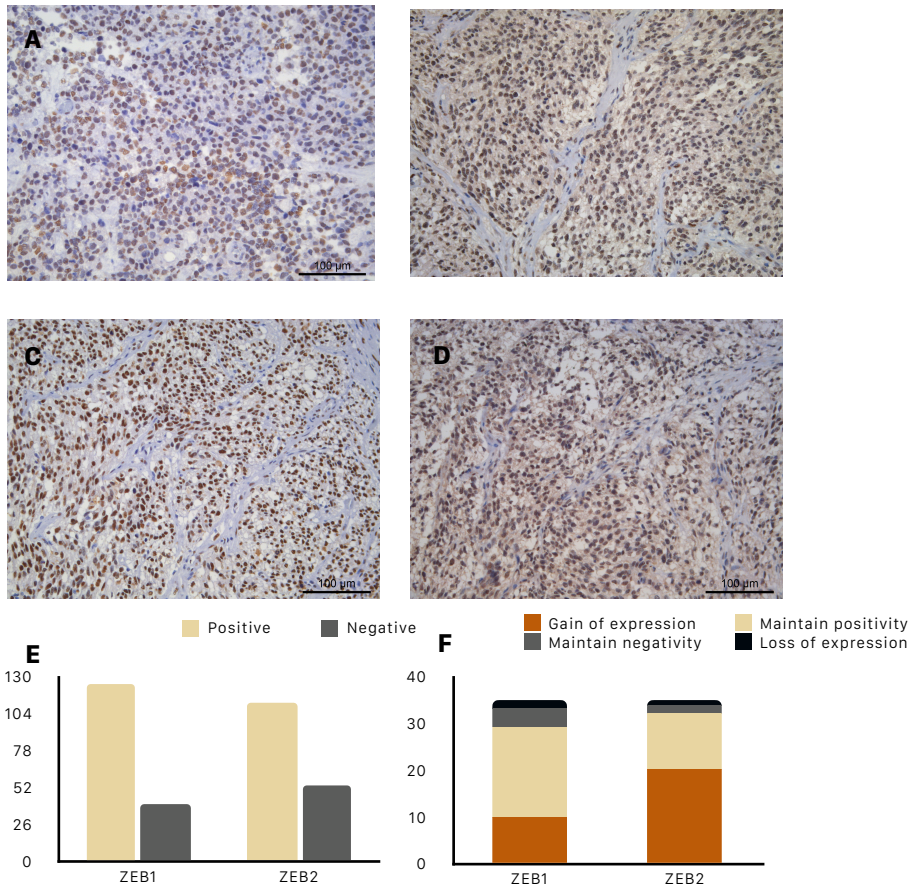


Figure 1. ZEB1 and ZEB2 expression in Glioblastoma. Example of ZEB 1 **(2A and 2C)** and ZEB2 **(2B and 2D)** expression in a primary and match-recurrent case, respectively (20x amplification). **(2E)** ZEB1 (n=125, 75,7%) and ZEB2 (n=111, 67,2%) expression in initial GBM. **(2F)** Expression of ZEB1 and ZEB2 considering match-recurrent cases.

3.2. ZEB1 and ZEB2 correlates with cadherin expression profiles

Upon evaluating the expression of ZEB1 and ZEB2, we correlated these findings with the data previously obtained for classical cadherins (E-, P- and N-cadherins) in primary and recurrent tumors [20]. N-cadherin expression was the most prevalent, present in 80% of the tumors and was uniformly distributed across ZEB1 and ZEB2 positive tumors **(Table I)**. E-cadherin expression showed a decline in ZEB2 positive cases (p=0.069). In particular, E-cadherin expression was significantly decreased in tumors expressing only ZEB2 (ZEB2+) expression (p=0.001) and significantly increased in tumors without ZEB1 or ZEB2 (ZEB1-ZEB2-) (p=0.011). Similarly, a trend suggesting a significant association between P-cadherin and ZEB1 expression was observed (p=0.064).

Table I. ZEB1/ZEB2 expression and Cadherin expression in Glioblastoma.

	GBM series	ZEB1		ZEB2		ZEB1+ZEB2+		ZEB1+		ZEB2+		ZEB1-ZEB2-	
	n (%)	n	p-value	n	p-value	n	p-value	n (%)	p-value	n	p-value	n	p-value
	165	125		111		89		36		20		20	
Ncad, n (%)	134 (81,2%)	103 (76,8%)	0.490	88 (65,67%)	0.362	72 (53,7%)	0.911	31 (23,13%)	0.395	14 (10,4%)	0.171	17 (12,7%)	0.644
Ecad, n (%)	72 (43,6%)	56 (77,8%)	0.594	43 (59,7%)	0.069	41 (56,9%)	0.496	15 (20,8%)	0.788	2 (2,8%)	**0.001**	14 (19,4%)	**0.011**
Pcad, n (%)	66 (40%)	55 (83,3%)	0.064	47 (71,2%)	0.379	41 (62,1%)	0.085	14 (21,2%)	0.878	4 (6,1%)	0.051	7 (10,6%)	0.629
Cadherin subgroups			0.014		**0.018**		**<0.001**		0.343		**0.002**		**0.032**
Ncad, n (%)	42 (25,5%)	34 (81%)	0.363	33 (78,6%)	0.071	26 (62%)	0.233	8 (19%)	0.617	7 (16,7%)	0.299	1 (2,38%)	**0.025**
Ecad, n (%)	6 (3,6%)	5 (83,3%)	0.659	5 (83,3%)	0.393	4 (66,7%)	0.527	1 (1,51%)	0.757	1 (1,51%)	0.730	0	0.357
Pcad, n (%)	7 (4,2%)	7 (100%)	0.126	7 (100%)	0.059	7 (100%)	**0.012**	0	0.155	0	0.318	0	0.318
Ecad/Ncad, n (%)	36 (21,8%)	26 (72,2%)	0.576	17 (47,2%)	**0.004**	16 (44,4%)	0.198	10 (27,8%)	0.330	1 (2,7%)	0.052	9 (25%)	**0.007**
Pcad/Ncad, n (%)	29 (17,6%)	22 (75,9%)	0.988	19 (65,5%)	0.824	12 (54,5%)	0.137	10 (34,5%)	0.07	5 (17,2%)		2 (6,9%)	0.345
Ecad/Pcad, n (%)	6 (3,6%)	6 (100%)	0.158	6 (100%)	0.082	6 (100%)	**0.021**	0	0.357	0	0.357	0	0.357
Triple positive, n (%)	25 (15,2%)	20 (80%)	0.591	16 (64%)	0.705	16 (64%)	0.276	4 (16%)	0.448	0	**0.044**	5 (20%)	0.192
Triple negative, n (%)	14 (8,5%)	5 (35,7%)	**<0.001**	8 (57,1%)	0.398	2 (14,3%)	**0.002**	3 (21,4%)	0.971	6 (42,9%)	**<0.001**	3 (21,4%)	0.267

Considering the cadherin expression subgroups, these further clarified the association between cadherins and ZEB **(Table I)**. Isolated expression of N-cadherin was observed in up to a quarter of the tumors, with higher expression rates in tumors positive for ZEB1 and/or ZEB2 expression. Notably, N-cad expression was found only one case of tumors lacking ZEB1/ZEB2 (ZEB1-ZEB2-) expression (p=0.025).

Consistent with the results observed for E-cadherin, the Ecad/Ncad subgroup was significantly less frequent in ZEB2-expressing tumors (p=0.004) and more prevalent in tumors lacking ZEB1/ZEB2 expression (ZEB1-ZEB2-) (p=0.007). The Pcad/Ncad subgroup was primarily associated with isolated ZEB1 expression, which showed a tendency towards significance (p=0.07). Finally, the subgroups with isolated P-cadherin expression and E-cad/P-cad were consistently observed in tumors with combined ZEB1+ZEB2+ expression (p=0.012 and 0.021, respectively).

3.3. Tumoral PD-L1 associates with ZEB1 expression

The median lymphocyte count in this GBM series was 1220 cells/µL and patients with lymphopenia (lymphocyte counts <1000 cells/uL) exhibited a significantly worse overall survival (p=0.049). Although not statistically significant, we observed that patients with combined ZEB1+ZEB2+ expression had the lowest median lymphocyte counts, just on the threshold of lymphopenia (1070 cells/µL). In contrast, higher lymphocyte counts were observed in patients with isolated ZEB2 (ZEB2+) expression or without ZEB1/ZEB2 expression (ZEB1-ZEB2-) **(Table II)**.

The median value of NLR in our series was 8.658 and we confirmed that a NLR >6 stratified patient prognosis (p=0.028). Intriguingly, we found higher medians for both ZEB1 (p=0.059) and ZEB2 (p<0.001), with the latter reaching statistical significance. When evaluating ZEB1/ZEB2 expression subgroups no significant differences were observed, although higher NLR ratios were observed in patients with tumors with combined ZEB1+ZEB2+ expression. The only

subgroup with a median inferior to our prognosis-associated cut-off (>6) was the one without ZEB1/ZEB2 (ZEB1-ZEB2-) expression, with a median value of 5.29 **(Table II)**.

TABLE II. ZEB1/ZEB2 expression and immunosuppression in Glioblastoma.

	GBM series	ZEB1		ZEB2		ZEB1+ZEB2+		ZEB1+		ZEB2+		ZEB1-ZEB2-	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value
	165	125		111		89		36		20		20	
Lymphocyte count (median, cells/µL)	1220	1120	0.208	1130	1,00	1070	0.472	1150	0.645	1620	0.175	1450	0.492
NLR (median)	8,658	11,317	0.059	11,199	**<0.001**	11,375	0.332	7,737	0.419	7,469	0.424	5,29	0.106
PD-L1	48 (29,1%)	42 (33,6%)	**0.024**	34 (30,6%)	0.532	28 (31,5%)	0.468	14 (38,9%)	0.143	5 (25%)	0.667	1 (5%)	0.011

Finally, we identified a significant correlation between PD-L1 expression and ZEB-1 expression (p=0.024). In tumors lacking ZEB1/ZEB2 (ZEB1-ZEB1-) expression, tumoral PD-L1 positivity was rare, occurring in only 1 case (5%) (p=0.011) **(Table II)**.

Considering the association between ZEB1/ZEB2 expression with both PD-L1 and classical cadherins expression subgroups, we still hypothesized that a correlation may exist between the latter two **(Table III)**. Indeed, we found that PD-L1 expression rates were significantly higher in cases positive for P-cadherin (p<0.001). In addition, when analyzing cadherin expression subgroups, PD-L1 expression rates were higher (>50%) in tumors with isolated Ecad (p=0.053) and Pcad/Ncad co-expression (p<0.001). Lower PD-L1 expression rates were observed in tumors with Ecad/Ncad co-expression (<20%, p=0.024). Concordantly with these results, patients with tumors exhibiting Pcad/Ncad co-expression had the lowest lymphocytic counts (p=0.054) and highest NLR values (p=0.047).

3.4. ZEB2 expression correlates with patients' survival outcomes

Survival analysis was used to correlate ZEB1 and ZEB2 expression with GBM patients' prognosis.

Table III. Cadherin expression and PD-L1 expression

	GBM series	Lymphocyte count (median, cells/µL)		NLR (median)		PD-L1	
			p-value		p-value	n	p-value
	n=313					n=96 (30,7%)	
Ncad, n (%)	255	1250	0.474	6,5	0.837	75 (78,1%)	0.311
Ecad, n (%)	97	1450	0.189	4,812	0.067	27 (75%)	0.532
Pcad, n (%)	65	1220	0.390	9,1	0.283	32 (33%)	**<0.001**
Cadherin subgroups			0.662				
Ncad, n (%)	137	1450	0.730	6,86	0.519	36 (26%)	0.137
Ecad, n (%)	4	1460	0.606	7,469	0.944	3 (75%)	0.053
Pcad, n (%)	8	1260	0.885	9,096	0.934	4 (50%)	0.230
Ecad/Ncad, n (%)	67	1620	0.523	4,812	0.130	13 (19,4%)	**0.024**
Pcad/Ncad, n (%)	29	900	0.054	12,41	**0.047**	17 (58,6%)	**<0.001**
Ecad/Pcad, n (%)	6	1340	0.925	10,11	0.744	3 (50%)	0.300
Triple positive, n (%)	22	1220	0.727	5,339	0.720	8 (36,4%)	0.119
Triple negative, n (%)	40	1390	0.316	7,343	0.418	10 (25%)	0.405

In particular, ZEB2 expression was found in tumors with a shorter time to recurrence (median 3 months, log rank test, p=0.002) **(Figure 2B)** and overall survival (median 10 months, log rank test, p=0.019) **(Figure 2E)**. No significant differences in survival were observed for ZEB1 positive cases, with median progression-free survival (PFS) of 5 and overall survival (OS) of 11 months **(Figure 2A and 2D)**.

Evaluation of survival based on ZEB1/ZEB2 subgroups revealed a significant stratification of PFS (p=0.003) **(Figure 2C)**. The worst prognosis was observed in patients with tumors with isolated ZEB2 expression (ZEB2+) (median 1 month) (p=0.016), followed by tumors with combined expression of ZEB1+ZEB2+ (median 4 months) (p=0.015) **(Figure 2C)**. Tumors lacking ZEB1/ZEB2 expression (ZEB1-ZEB2-) exhibited significantly longer times to recurrence

(median 7 months) (p=0.025) **(Figure 2C)**. Overall survival statistics showed significantly shorter survival for ZEB1+ZEB2+ tumors (median 10 months, p=0.027) **(Figure 2F)**.

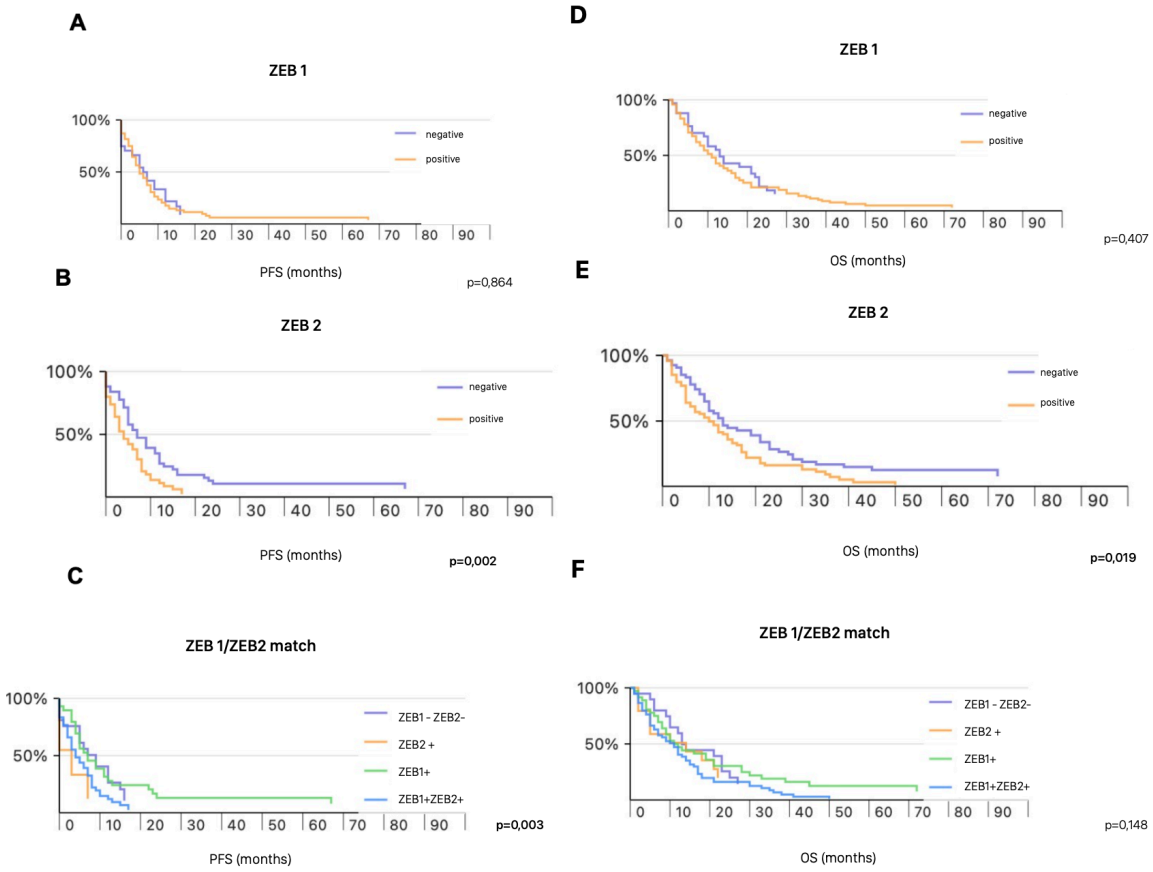


Figure 2. Survival analysis for ZEB1 and ZEB2 expression. Time to recurrence **(2A)** and overall survival **(2D)** of tumors with ZEB1 expression did not show significant differences, while ZEB2 expression tumors associate with worse PFS **(2B)** and OS **(2E)**. ZEB1/ZEB2 expression subgroups stratifies time to recurrence **(2C)**, in particular ZEB1+ZEB2+ were statistically associated with a worse overall survival (p=0.027) **(2F)**.

4. Discussion

The present study investigates the correlation between ZEB1/ZEB2 transcription factors and PD-L1 expression in the context of an EMT-like program and its role on immune evasion in GBM. Emerging data supports the interplay between the EMT and immune evasion mechanisms across systemic cancers [14,15,25]. Specifically, ZEB1 has been linked to T-cell exclusion and immune escape in melanoma cells [26] and has been shown to regulate PD-L1 expression in other systemic cancers [16,27]. Several patient cohorts, including ours, have documented significantly higher PD-L1 expression in tumors with mesenchymal phenotypes [24,28–30]. However, the regulatory dynamics of these mechanisms in GBM remains less understood.

To address our research question, we documented ZEB1 and ZEB2 expression in GBM samples. Our results demonstrate that ZEB1 and ZEB2 are expressed in a high proportion of GBM cases - 74% and 57% of the tumors, respectively. Upon tumor recurrence, expression rates significantly increase, with a noticeable gain in ZEB2 expression in over half of matched primary-recurrent tumors. The observed increase in expression for both ZEB1 and ZEB2 suggests that these transcription factors play a critical role in therapy resistance and tumor recurrence. Indeed, activation of ZEB-mediated pathways has been proposed as a mechanism of treatment resistance in other tumors [31], and a recent study implicated ZEB1 as a driver for bevacizumab resistance in glioblastoma cells [32].

All tumors with a predominant mesenchymal phenotype exhibited ZEB1 expression, suggesting its central role in GBM mesenchymal state, consistent with reports in various cancers [33–36] and more recently in GBM [18,19,37,38]. Despite this, the clinical significance of ZEB transcription factors in gliomas remains controversial [39]. Survival analysis in our cohort associates ZEB2-expressing tumors with worse progression-free and overall survivals. This aligns with previous studies identifying ZEB2 as a marker of poor prognosis [40,41], including two studies specifically in GBM [42,43].

The relationship between ZEB1/ZEB2 and classical cadherins is central to the EMT process. Indeed, ZEB1 and ZEB2 transcription factors condition the cadherin switch through E-cadherin expression repression and transcriptional activation of N-cadherin [36,44,45]. In GBM, ZEB1 has been implicated in the dynamics of N-cadherin [19,46]. P-cadherin, another epithelial cadherin, has been associated with the intermediate phenotype in systemic cancers [47,48] and ZEB1-mediated upregulation of P-cadherin has been linked to increased tumor aggressiveness and poor prognosis[49].

Although without statistical significance, our results present a tendency for an association between E-cadherin and P-cadherin expression according to ZEB2 and ZEB1 expression, respectively. Qi et al. concordantly found that ZEB2 downregulation resulted in the restoration of E-cadherin expression in gliomas cell lines [21]. On the other hand, ZEB1 expression appears in early hybrid states and is conserved throughout the mesenchymal stage [50], and thus the association between ZEB1 and P-cadherin interestingly brings to question whether ZEB1 could be also involved in EMT intermediate stages also in GBM. Furthermore, in melanoma, a high ZEB2/low ZEB1 is associated with proliferation, while low ZEB2/high ZEB1 favors invasion and migration, thus suggesting a critical role for the ZEB2/ZEB1 ratio in tumor phenotypic state [51]. In addition, it supported the existence of several intermediate states with ZEB1/ZEB2 co-expression. Indeed, these intermediate or hybrid states have gain considerable interest in the last decade. Referred to as epithelial-mesenchymal plasticity (EMP), it denotes the co-existence epithelial and mesenchymal characteristics, leading to increased tumor plasticity and an advantage in cancer pathogenesis [10,50,52–54].

Nevertheless, markers of EMT states in GBM are largely unknown. We had previously hypothesized how classical cadherins reflect different EMT stages in GBM, using the same large cohort of patients with detailed neuropathological evaluation. Analysis of ZEB1 and ZEB2 activation in these samples provided additional insight into our hypothesis. Most interesting was the evaluation of ZEB1/ZEB2 co-expression according to cadherin

subgroups. The Ecad/Ncad subgroup, which we have previously associated with the epithelial phenotype and with a survival benefit, significantly associates with the absence of these transcription factors (ZEB1-/ZEB2-). In contrast, the Pcad/Ncad subgroup, which we proposed as a marker of the mesenchymal stage in GBM, is associated with ZEB1 positivity. Finally, we had previously hypothesized that the isolated expression of epithelial cadherins, Pcad and Ecad/Pcad subgroups, reflected EMP in GBM, denoted by tumors with higher plasticity and poor outcome. Indeed, these subgroups were statistically associated with the combined ZEB1+/ZEB2+ expression. Taken together, our results clearly show that ZEB1 and ZEB2 activation associates with different cadherin expression subgroups. Furthermore, we present compelling evidence that these subgroups denote not only differences in ZEB1/ZEB2 expression, but mostly distinct EMT states in GBM.

We then decided to evaluate initial lymphocytic counts, neutrophil-to-lymphocyte ratio (NLR) and GBM PD-L1 expression according to ZEB1/ZEB2 expression. A notable finding of this study is the significant correlation between ZEB1 expression and PD-L1 positivity in GBM, highlighting the putative dual role of ZEB1 in both EMT and immune escape mechanisms. The ZEB1-ZEB2- subtype, which is significantly associated with the predominant epithelial phenotype, exhibited higher lymphocyte counts and lower NLR values and significantly lower PD-L1 expression rates (5%). In contrast, PD-L1 expression rates were similar among ZEB1+ZEB2+, ZEB1+ and ZEB2+ subgroups (25-38%), supporting the notion that immune evasion mechanisms are early activate in the EMT program, and not only in the mesenchymal stage [16].

Our data on cadherins expression also showed an increase in PD-L1 expression in tumors positive for P-cadherin. This result reflects the higher expression of PD-L1 in the Pcad/Ncad (59%), Pcad (50%) and Ecad/Pcad (50%) cadherin subgroups, and hence an interconnection between immune evasion and intermediate/mesenchymal EMT states in GBM. Furthermore, tumors with Pcad/Ncad co-expression exhibited not only higher rates of PD-L1 expression, but also important trends for marked lymphopenia, indicating profound systemic

immunosuppression, and higher levels of neutrophil-to-lymphocyte ratios (NLR), suggesting higher systemic inflammation.

Thus, considering the continuum of an EMT program in GBM, we propose the identification of three different stages. One without ZEB expression (ZEB1-ZEB2-), which is evidenced by co-expression of Ecad/Ncad and tumors often exhibiting an epithelial phenotype. We have previously correlated this stage with a survival benefit. A second one marked by combined ZEB1+ZEB2+ expression, frequent expression of epithelial cadherins (Pcad and Ecad/Pcad subgroups) and phenotypic variability. In this stage, PD-L1 expression rates increase, as does systemic immunosuppression (lower lymphocyte counts) and inflammation (higher NLR). The combination of increasing immunosuppression and high tumoral plasticity explain the associated poor outcomes - ZEB1+ZEB2+ co-expressing tumors exhibited shorter times for recurrence in our analysis. Third, a mesenchymal stage, reflected by ZEB1+ expression and Pcad/Ncad co-expression, with a significant upregulation of PD-L1 expression and profound systemic immunosuppression/inflammation. Other subgroups position is less clear, although they most probably reflect the variety of hybrid phenotypes in GBM. For instance, contrary to Ecad/Ncad, isolated Ecad tumors most frequently exhibit ZEB1+ZEB2+ co-activation, PD-L1 up-regulation (present in 75% of tumors) and a poor patient outcome. Epithelial differentiation is rare in GBM, and the differences in associated prognosis of these two subgroups led us to hypothesize that although phenotypically similar, they underly different cellular dynamics: isolated Ecad likely marks for EMP with concomitant activation of mechanisms of immune evasion, while Ecad/Ncad probably associates with the activation of underlying MET (mesenchymal-to-epithelial transition) pathways. Similarly, isolated Ncad tumors, the most frequent subgroup in GBM, also most frequently associates with ZEB1 and ZEB2 expression, particularly with the combined ZEB1+ZEB2+ subgroup, which may also point to a partial EMT state. Indeed, GBM is notorious for its local and systemic immunosuppression, treatment resistance and inevitable tumor progression, as well as local high proliferation, aggressiveness and invasive capacity. The co-existence of all these characteristics evokes the

activation of an EMP program [35,54–57]. Future research should focus on elucidating the precise molecular mechanisms by which ZEB1 and ZEB2 regulate cadherin expression in GBM. In conclusion, we herein provide compelling evidence that ZEB1 and ZEB2 play integral roles in an EMT-like process in GBM, which both potentiates and is potentiated by mechanisms of immune evasion. Altogether, these mechanisms contribute to treatment resistance and tumor progression. Furthermore, cadherin expression profiles are valuable indicators to predict different stages of EMT activation and concordantly degrees of immunosuppression.

5. References

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6. Supplementary data

Supplementary Table I. ZEB1/ZEB2 expression and correlation to tumor characteristics.

	GBM series	ZEB1		ZEB2		ZEB1+ZEB2+		ZEB1+		ZEB2+		ZEB1-ZEB2-	
	n (%)	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value	n (%)	p-value
	124 (100%)	92 (74,2%)		71 (57,3%)		57 (46%)		35 (28,2%)		13 (10,5%)		19 (15,3%)	
Papillary structures	9 (7,3%)	7 (7,6%)	ns	6 (8,5%)	ns	6 (10,5%)	ns	1 (2,9%)	ns	0	ns	2 (10,5%)	ns
Whorls	3 (2,4%)	3 (3,3%)	ns	2 (2,8%)	ns	2 (3,5%)	ns	1 (2,9%)	ns	0	ns	0	ns
Myxoid stroma	9 (7,3%)	6 (6,5%)	ns	4 (5,6%)	ns	2 (3,5%)	ns	4 (11,4%)	ns	1 (7,7%)	ns	2 (10,5%)	ns
Inflammation	47 (37,9%)	35 (38%)	ns	28 (39,4%)	ns	22 (38,6%)	ns	13 (37,1%)	ns	5 (38,5%)	ns	7 (36,8%)	ns
Calcifications	2 (1,6%)	2 (2,2%)	ns	1 (1,4%)	ns	1 (1,8%)	ns	1 (2,9%)	ns	0	ns	0	ns
Oligodend. component	8 (6,5%)	6 (6,5%)	ns	6 (8,5%)	ns	4 (7%)	ns	2 (5,7%)	ns	2 (15,4%)	ns	0	ns
Gemistocytes	16 (12,9%)	12 (13%)	ns	10 (14,1%)	ns	8 (14%)	ns	4 (11,4%)	ns	2 (15,4%)	ns	2 (10,5%)	ns
Glial component	124 (100%)	92 (100%)	ns	71 (100%)	ns	57 (100%)	ns	35 (100%)	ns	13 (100%)	ns	19 (100%)	ns
Mesenchymal component	40 (32,3%)	33 (35,9%)	ns	26 (36,6%)	ns	22 (38,6%)	ns	11 (31,4%)	ns	3 (23,1%)	ns	4 (21,1%)	ns
Epithelial component	28 (22,6%)	23 (25%)	ns	18 (25,4%)	ns	14 (24,56%)	ns	6 (17,1%)	ns	1 (7,7%)	ns	7 (36,8%)	ns
Predominant component													
Glial	115 (92,7%)	85 (92,4%)	ns	67 (94,4%)	ns	54 (94,7%)	ns	31 (88,6%)	ns	13 (100%)	ns	17 (89,5%)	ns
Mesenchymal	6 (4,8%)	6 (6,5%)	ns	4 (5,6%)	ns	3 (5,3%)	ns	3 (8,6%)	ns			0	
Epithelial	3 (2,4%)	1 (1,1%)	ns	0	ns	0		1 (2,9%)	ns			2 (10,5%)	**0.012**
Tumor recurrence	36	29 (80,6%)		32 (88,9%)		25 (69,4%)		4 (11,1%)		7 (19,4%)		0	

CHAPTER VII: General Discussion, Conclusions and Future Directions

1. General Discussion

Glioblastoma (GBM) remains one of the most lethal and enigmatic tumors. This rare, diffusely infiltrative cancer has a complex and poorly understood biology, partly attributed to limited sampling due to its location within the central nervous system (CNS). Current treatment strategies for GBM are based on pathogenic pathways effective on systemic cancers that, by overlooking the unique biological characteristics of GBM, have thus far created only modest results.

To study this disease, we have established a cohort of 313 GBM with appropriate patients' clinical records, imaging data and follow-up **(Chapter III)**. We validated our cohort for further analysis through the confirmation of previously established prognostic factors and determined similar survival times. We provided an in-depth analysis of GBM cases, while identifying key clinical and treatment characteristics, such as age, initial functional status (KPS) and extent of resection (EOR) as predictors of patient outcome. In addition, survival statistics in GBM encompass a poorly understood subgroup with early clinical deterioration, frequently due to intrinsic tumor aggressiveness. A systematic and detailed analysis of initial MRI features was conducted, documenting VASARI (Visually Accessible Rembrandt Images) characteristics, which established several features predictive of patient outcome. Our results emphasize the importance of integrating clinical (age, clinical presentation, KPS) and imaging characteristics (particularly cysts, multifocal disease, ependymal extension, bilateral contrast-enhancing lesions, deep white matter invasion, satellites, pial invasion, nodular enhancing tumor) to refine preoperative survival prognostication and guide treatment decision in GBM patients. Further studies are needed to improve these prognostic tools and validate their applicability in diverse clinical settings.

Given the actual relevance of immunotherapy and, particularly, PD-1/PD-L1 interactions in the immunological control of melanoma and epithelial cancers [1], we decided to investigate

GBM-associated local and systemic immunosuppression and inflammation **(Chapter IV)**. Our results clearly reflect important rates of immunosuppression upon initial diagnosis, as over half of the patients had lymphopenia previous to any treatment-induced side effect. We defined a neutrophil-to-lymphocyte ratio (NLR) value of 6 as a cut-off associated to patient prognosis, with values above reflecting higher levels of inflammation and immunosuppression. This data is highly relevant, since lymphopenia and systemic immunosuppression are frequently regarded as consequences of adjuvant treatment in GBM [2–5], but should otherwise be taken into consideration as important mechanisms of primary GBM. Local immunosuppression was evident by the frequent TIL location in the tumor periphery and PD-L1 expression in one third of the tumors. In addition, PD-L1 staining was significantly increased in tumor recurrence: 36% of initially PD-L1 negative primary cases became positive upon recurrence. As a summary, our main observations in this retrospective study were: i) GBM-associated immunosuppression (lymphopenia, high NLR, PD-L1 expression) significantly predicts poor patient prognosis; ii) TIL expression in the tumor periphery is associated with PD-L1 expression in 87,5% of the cases, which further suggests an immune-excluded phenotype, in which T cells are incapable of invading the tumor core; iii) PD-L1 positivity was more frequent in IDH and ATRX wild-type tumors, with areas of sarcomatous dedifferentiation. Moreover, PD-L1 increase upon tumor recurrence supports its importance in treatment resistance mechanisms.

Taken together, our results confirm the preponderance of GBM-induced immunosuppression and inflammation, particularly the significance of PD-1/PD-L1 axis in tumor pathogenesis and aggressiveness. PD-L1-associated prognosis appears to reflect, not only the capacity for tumor immune escape and the related TIL immune-excluded phenotype, but also mechanisms underlying treatment resistance. PD-1/PD-L1 therapies hold great promise in cancer treatment, however many unresolved questions still exist and mostly reflect the necessity to understand the intricacies of the mechanisms associated with PD-L1 expression. The higher percentage of PD-L1 expression in tumors with a sarcomatous component aligns

with similar studies in the literature, which propose GBM subtype-specific immune profiles [6–8] and, in particular, high PD-L1 expression in the mesenchymal subtype [9–12]. Moreover, these findings point to a relationship between tumor immune escape and the epithelial-mesenchymal transition (EMT) process, where tumor resistance to immune degradation may induce or perpetuate the activation of EMT pathways. On its turn, and thereby amplifying tumor aggressiveness, EMT activation may as well underlie the activation of immune escape mechanisms [13–18].

Driven by our group's expertise in cadherins, we initiated our study of an EMT-like program in GBM with the characterization of cadherins expression profiles in GBM **(Chapter V)**. The expression of cadherins, such as E-, P-, and N-cadherin, plays a significant role in the prognosis of various systemic cancers, particularly epithelial tumors, as the EMT program involves cadherin expression regulation based on cell-cell adhesion requirements [19,20]. This process is pivotal and reflected by the cadherin switch, E- to N- cadherin, which is consensually regarded as a marker of an EMT program activation in epithelial cancers [20–22]. There is nonetheless a knowledge gap regarding cadherin expression in non-epithelial tumors, including GBM.

To our knowledge, we present the largest patient cohort with characterization of cadherins' expression in this tumor type. We documented N-cadherin expression to be the most common in over 80% of GBM cases, followed by E-cadherin in about one-third of the tumors and P-cadherin in 20% of the cases. Upon tumor recurrence, P-cadherin and N-cadherin are the predominant cadherins being expressed and P-cadherin, in particular, increases its expression to 65,8%. This was in concordance with the survival statistics, denoting a worse prognosis for patients with P-cadherin positive tumors. Although with a striking result pointing to a clear role for P-cadherin in GBM biology, we questioned what was the significance of the two other more prevalent cadherins, E-cadherin and N-cadherin. For instance, our results showed that E-cadherin expression was commonly observed in temporal tumors with cysts, an epithelial differentiation and, contrary to what had been previously

documented [23,24], associated with a survival benefit. Our group had previously reported that the interplay between E- and P-cadherin influences their biological behaviour [25], and, therefore, we hypothesized that evaluation of the combined expression of these cadherins would clarify our results. In fact, analysis of the combined expression of E-, N- and P-cadherin improved the identification of biological-relevant subgroups, with distinct imaging, neuropathological and survival characteristics.

In brief, we identified the following subgroups: i) isolated N-cadherin expression represents the most common subgroup of GBM; ii) E- and N- cadherin co-expression identifies a subgroup with longer survival (PFS; OS) and the most common subgroup among long term survivors. These tumors have temporal location and cysts and frequently present an epithelial differentiation; iii) P- and N-cadherin co-expression predicts one of the subgroups with worse prognosis, the most common subgroup upon tumor recurrence. These tumors show a predominant mesenchymal differentiation and an association to the mesenchymal subtype in TCGA data; iv) Ecad/Pcad co-expression and isolated Pcad expression associate with frequent tumor heterogeneity and MRI characteristics associated with tumor aggressiveness, which is corroborated by the poorest patient outcomes; v) isolated Ecad expression also associates with epithelial-differentiated tumors; however, it points to a subgroup of patients with a worse prognosis.

As it is represented in **Figure 1**, isolated Ncad expression and Ecad/Ncad co-expression were the most common profiles in GBM, and Ecad/Ncad co-expression frequently associates with a biological signature associated to an epithelial differentiation and to a survival benefit.

Nevertheless, the findings on isolated Ecad subgroup prevent us from conclusively assuming that the epithelial differentiation in GBM corresponds to a favorable phenotype. For the mesenchymal state, we identified Pcad/Ncad co-expression as a biomarker. The EMT concept defines a continuum between epithelial and mesenchymal stages, with the presence of intermediate or hybrid stages of differentiation, referred to as epithelial-mesenchymal plasticity (EMP). These are stages with high plasticity, stem-like properties and increased

invasion/dissemination capacity [26–29]. According to our data, the expression of isolated Pcad and Ecad/Pcad co-expression most probably reflects the presence of some of these intermediate phenotypes. Importantly, the intrinsic characteristics of GBM, namely its marked intratumoral heterogeneity, aggressiveness and local invasive capacity, led us to speculate that other cadherins expression subgroups could also reflect EMP, including the epithelial-differentiated isolated Ecad subgroup.

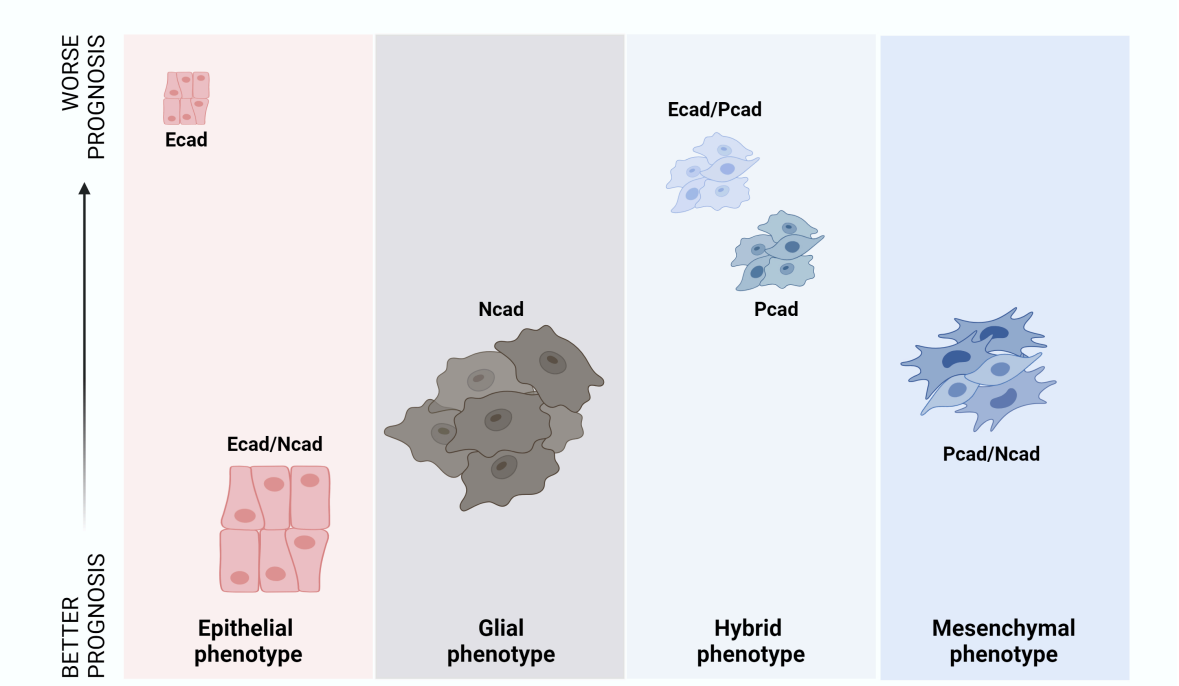

Figure 1. Co-expression of E-, N- and P-cadherin in Glioblastoma and its correlation to tumor differentiation and prognosis. Tumors with isolated Ncad expression were the most common. When N-cadherin was co-expressed with E-cadherin (Ecad/Ncad), tumors frequently exhibit an epithelial differentiation and patients significantly had the best outcomes. On the contrary, isolated Ecad stained tumors, although also with frequent epithelial differentiation, exhibit with the worst prognosis. Similar to isolated Ecad, the expression of other epithelial cadherins, such as the Ecad/Pcad and Pcad profiles, point to tumors with worse survival outcomes. These tumors commonly exhibited various phenotypic components. Finally, Pcad/Ncad co-expression appears to denote tumors with a mesenchymal phenotype and is the most frequent subgroup upon tumor recurrence.Created with BioRender[i].

i Noronha C, Ribeiro AS, Carvalho R, Mendes N, Reis J, Faria C, Taipa R, Paredes J. Cadherin expression profiles define glioblastoma differentiation and patient prognosis. Cancers, 16(13): 2298, 2024

In order to deeply explore our concept, we considered that in the context of a classical EMT, cadherins expression is primarily regulated by ZEB1/ZEB2 transcription factors [30–34]. Thus, the results obtained in cadherins analysis **(Chapter V)** anticipated a role for ZEB1/ZEB2 in GBM **(Chapter VI)**. Indeed, high expression rates of ZEB1 (74%) and ZEB2 (57%) were found in our cohort, with a significant increase of their expression upon tumor recurrence, particularly for ZEB2 (88,9%). As expected, ZEB2 was a repressor of E-cadherin expression [35] and all tumors with a predominant mesenchymal phenotype were positive for ZEB1, confirming its relationship with the mesenchymal phenotype in GBM [36–38]. The importance of ZEB1/ZEB2 expression in GBM was further confirmed by the identification of ZEB2 as a significant predictor of prognosis. An interesting result was the association between P-cadherin expression and ZEB1. P-cadherin has been proposed as a marker of EMP [39,40] and this association suggests that, although ZEB1 facilitates the transition to a mesenchymal phenotype in GBM, it is still observed in partial EMT phenotypes. The evaluation of ZEB1/ZEB2 co-expression further suggested that this association between P-cad and ZEB1 is more frequent with concomitant expression of ZEB2 (ZEB1+ZEB2+ subgroup).

Thus, the integration of cadherin and ZEB1/ZEB2 expression subgroups, can be summarized as follows: i) the absence of ZEB1/ZEB2 (ZEB1-ZEB2-) associates with the epithelial phenotype and Ecad/Ncad co-expression; ii) ZEB1+ expression is associated with half of the predominant mesenchymal tumors and with P- and N-cadherin co-expression; iii) combined ZEB1+ZEB2+ expression is observed in all isolated Pcad and Ecad/Pcad co-expressing tumors, and in the majority of tumors with isolated Ecad (67%) and isolated Ncad (62%). This subgroup presented the worse survival outcomes.

We therefore provide compelling evidence that ZEB1/ZEB2 are key contributors to GBM aggressiveness and underly changes that can be integrated in the EMT paradigm. We further contribute with data revealing that ZEB1/ZEB2 expression in GBM is reflected by cadherin co-expression subgroups, which we anticipate may further improve subgroup definition in GBM and prognosis prediction.

Building on our idea of the EMT paradigm in GBM, we pursued to evaluate if the interplay between the EMT and the immune system described for systemic tumors [13,15], specifically regarding ZEB1 and ZEB2 expression [16,41–43], was also definable in our series. We evaluated systemic immunosuppression and inflammation through previously evaluated and validated biomarkers, initial lymphocytic count and NLR; and local immunosuppression through PD-L1 expression. A clear correlation between ZEB1 and PD-L1 positivity was observed. In addition, in tumors without ZEB1-ZEB2- expression a low rate of PD-L1 positivity was observed (5%), the median lymphocyte count was normal and NLR was under the cut-off value that has been associated with poor outcomes in GBM [44–46], contrary to what was observed for tumors with ZEB1/2 expression. Furthermore, as we theorized that cadherin expression subgroups could improve the identification of relevant GBM subgroups, we evaluated local and systemic immunosuppression among them. Indeed, this provided important information to our model, that we herein summarize and represent in **Figure 2:**

1. PD-L1 expression is significantly higher in the Pcad/Ncad subgroup, which is also accompanied by a profound systemic immunosuppression and high inflammation. The Pcad/Ncad subgroup also correlates with ZEB1 expression subgroup and the mesenchymal phenotype. We propose this subgroup as indicative of the GBM mesenchymal subtype.
2. Pcad (50%), Ecad/Pcad (50%) and Ecad (75%) also exhibit high rates of PD-L1 expression, although the markers for a systemic immunosuppression are less obvious. Pcad and Ecad/Pcad expressing tumors were always ZEB1+ZEB2+, and Ecad tumors were ZEB1+ZEB2+ positive in 67% of the tumors. Although Ecad tumors frequently exhibited an epithelial differentiation, GBM is not an epithelial tumor and, thus, we hypothesize that Ecad expressing tumors may align with Pcad and Ecad/Pcad tumors, collectively representing a hybrid EMT phenotype or EMP.
3. The lowest PD-L1 expression was significantly observed in Ecad/Ncad tumors, with concordant high peripheral lymphocyte counts and a NLR associated with low

inflammation. Ecad/Ncad tumors frequently exhibit an epithelial-like differentiation, associate with the absence of ZEB expression (ZEB1-ZEB2-) and exhibit a clear survival benefit. We integrate it in the concept of an epithelial differentiation in GBM, perhaps denoting activation of MET pathways.

4. Finally, isolated Ncad tumors, the most frequent subgroup, does not demonstrate a particular immune trend, while they most frequently exhibit combined ZEB1+ZEB2+ (62%) positivity. Altogether, and especially considering the inherent characteristics of GBM, we propose that the isolated Ncad tumors, and hence the majority of GBM, denote an EMP.

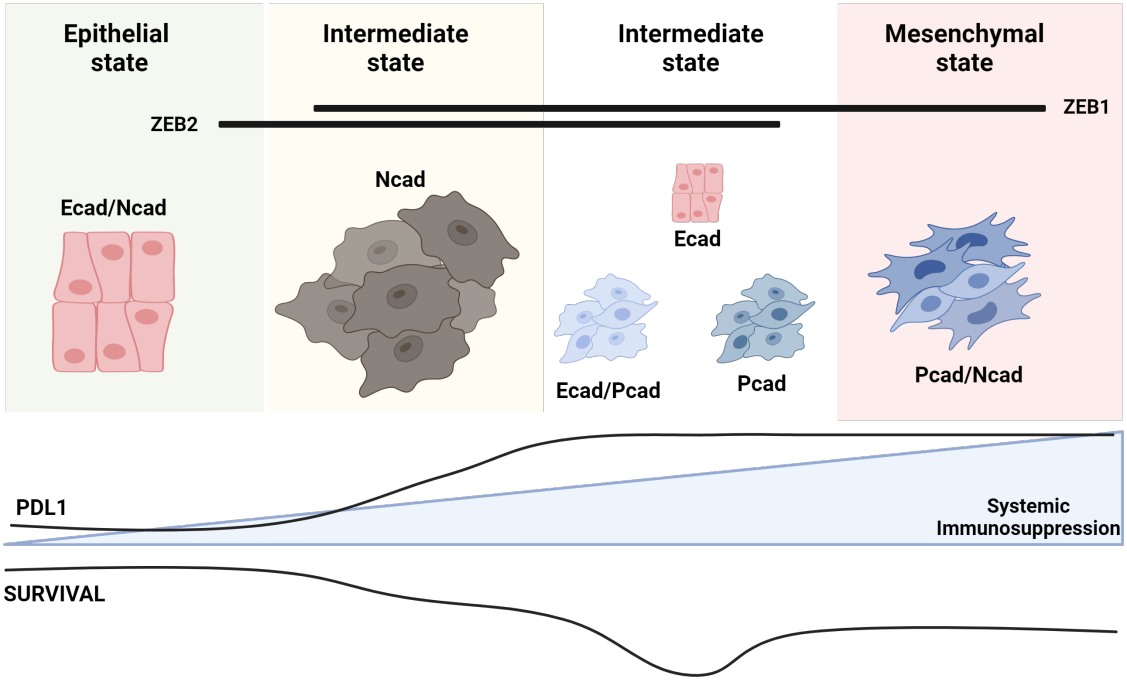

Figure 2. Interplay between cadherin subgroups, ZEB1/2 expression and immunosuppression status in Glioblastoma. The mesenchymal phenotype in GBM is represented by Pcad/Ncad co-expression, ZEB1+ expression, PD-L1 upregulation and significant systemic immunosuppression. Various intermediate phenotypes exist in GBM, denoting its heterogeneity. These include the most common GBM profile, isolated Ncad expression, but also the subgroups with epithelial cadherins expression (Ecad, Pcad, Ecad/Pcad), which are associated with poor patient outcomes. Most commonly, ZEB1+ZEB2+ co-activation is observed in this stage. Finally, we identified the rare epithelial differentiation, with Ecad/Ncad expression, low PD-L1 expression and immunosuppression, and absence of ZEB1-ZEB2- expression. Created with BioRender.

Importantly, many interesting questions arise from the results obtained under this thesis. Future research should focus on elucidating the precise molecular mechanisms by which ZEB1/ZEB2, cadherins and PD-L1 interact and regulate one another. Converting the apparent intricate relationship between tumor immune evasion and an EMT-like program into a potential therapeutic target, should motivate further understanding of the EMT-immune evasion axis in GBM. Moreover, one crucial step to guarantee therapeutic benefit is to identify predictive biomarkers of treatment response. We anticipate that, with careful validation of our findings, cadherins hold great promise in guiding subgroup stratification and treatment decision in GBM patients.

2. Concluding Remarks and Future Directions

In this work, we present compelling evidence supporting the involvement of an EMT program in GBM, and that its interplay with immune evasion mechanisms potentiates GBM aggressiveness. Furthermore, our research introduces the novel concept that cadherin expression subgroups are reliable predictors of this interplay, and that the dynamics of cadherin expression in GBM differ from those in other cancers.

As with any research project, many ideas and questions are herein left unanswered. The choice to first describe these mechanisms in a large GBM patient series, and particularly to stain every available sample from each patient, was based on GBM significant heterogeneity and the important lack of knowledge regarding its underlying pathogenesis. With the robust data collected, we now have the biological rationale to pursue mechanistic studies. In addition, we developed a remarkable collaboration for MRI features evaluation that, although goes unnoticed in this thesis, will undoubtedly continue to improve the identification of MRI biomarkers.

Another interesting chapter, not concluded in time for this thesis, is the integration of GBM microstructure with the results achieved thus far. GBM must overcome a mechanically challenging microenvironment, characterized by a dense extracellular matrix. The activation of mechanosensitive biochemical pathways has been shown to induce the EMT in other tumors, while GBM stiffness has been associated to tumour aggressiveness and prognosis.

On a personal note, I conclude this thesis by sharing that becoming a physician-scientist has been a long-held ambition. While it initially stemmed from my natural curiosity during medical school and in the early days of my residency, it quickly grew to reflect my helplessness in confronting the inevitable outcomes faced by our patients. My lab work, my continuous reading and the incessant questioning of this devastating disease; they have set the tone for what I can only describe as a greatly rewarding journey with immense professional and personal growth. My curiosity, I am confident it will continue to write the many chapters ahead.

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Review

Cadherin Expression and EMT: A Focus on Gliomas

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Abstract: Cadherins are calcium-binding proteins with a pivotal role in cell adhesion and tissue homeostasis. The cadherin-dependent mechanisms of cell adhesion and migration are exploited by cancer cells, contributing to tumor invasiveness and dissemination. In particular, cadherin switch is a hallmark of epithelial to mesenchymal transition, a complex development process vastly described in the progression of most epithelial cancers. This is characterized by drastic changes in cell polarity, adhesion, and motility, which lead from an E-cadherin positive differentiated epithelial state into a dedifferentiated mesenchymal-like state, prone to metastization and defined by N-cadherin expression. Although vastly explored in epithelial cancers, how these mechanisms contribute to the pathogenesis of other non-epithelial tumor types is poorly understood. Herein, the current knowledge on cadherin expression in normal development in parallel to tumor pathogenesis is reviewed, focusing on epithelial to mesenchymal transition. Emphasis is taken in the unascertained cadherin expression in CNS tumors, particularly in gliomas, where the potential contribution of an epithelial-to-mesenchymal-like process to glioma genesis and how this may be associated with changes in cadherin expression is discussed.

Keywords: cadherins; EMT; gliomas

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1. Cadherins: The Main Regulators of Cell-Cell Adhesion

Cell-cell contact and adhesion are indispensable mechanisms for tissue-specific tasks and homeostasis by defining cell polarity and tissue compartmentalization [1,2]. Amongst the several families of adhesion molecules, the major one is the superfamily of cadherins (named for "calcium-dependent adhesion"), which are transmembrane proteins involved in the formation of adherens junctions (AJ) [3]. Cell-cell adhesion is mediated by extracellular cadherin domains, which function in a calcium-dependent way. In contrast, the intracellular cytoplasmic tail associates with numerous adaptor and signaling proteins collectively referred to as the cadherin adhesome [3]. The cadherin superfamily includes classical

cadherins, protocadherins, desmogleins, desmocollins, and others [3], which structurally share extracellular cadherin repeats.

Classical cadherins include epithelial (E-), neuronal (N-), placental (P-), and retinal (R) cadherins, which were the first members of the superfamily to be identified (CDH1, CDH2, CDH3, and CDH4, respectively). They are calcium-binding proteins, characterized by an ectodomain containing five extracellular cadherin repeats, a transmembrane domain, and a cytoplasmic domain with highly conserved binding sites for p120-catenin and β-catenin [4]. The cytoplasmic domain also participates in cell-cell adhesion by stabilizing the cadherin/catenin complex at the membrane and binding this complex to the actin cytoskeleton. Moreover, the diversity of cadherin-binding molecules potentiates the crosstalk between cadherins and other cellular systems, pointing to their key role as regulators of cellular behavior. Indeed, although the cytoplasmic tails of different classical cadherins bind the same cytosolic proteins, and cadherin/catenin complexes appear to be similar in different cellular contexts, cadherin-mediated signaling is nevertheless highly dependent on the cellular context. It regulates a wide range of normal physiological processes, including embryo development, apoptosis, gene expression, cell proliferation, differentiation, and migration [5–7].

Cadherins assemble into strong adhesive intercellular junctions with subtype specificities, mainly through homophilic connections. Cells expressing a given cadherin are thought to preferentially adhere to those expressing the same cadherin subtype since the expression of distinct cadherins is involved in cell sorting [8]. However, cadherins' expression in cells can also be heterogeneous, with cells expressing multiple cadherin subtypes, which leads to cadherin-mediated heterotypic adhesion [9,10]. Cadherin complexes are perceived as dynamic, undergoing cycles of assembly and disassembly [11,12]. It is the combination of qualitative and quantitative cadherin expression differences that most likely confers tissue-specific characteristics. These characteristics also include the number of shearing forces on cells [13], pointing to a biophysical basis for morphogenetic phenomena [14].

Cadherins play crucial roles during embryonic development and during the maintenance of adult tissues' normal architecture [15,16]. Thus, several human diseases result from compromised cadherin expression and function, including skin, cardiovascular and neuronal disorders, and cancer, as recently reviewed by Vestweber and colleagues [17].

2. Cadherins Expression in Normal Adult Tissues

The paradigm for cadherin function stems mostly from studies concerning E-cadherin, with a vast amount of literature documenting E-cadherin's impact on tissue architecture and morphogenesis [18,19]. Dynamic expression of E-cadherin was found to be a prerequisite for cell migration and morphogenesis during embryonic development [15]. A decrease in E-cadherin expression is observed, for instance, during gastrulation when mesoderm is formed [20] and in ectoderm during neurulation [21], while its re-expression is indispensable for skin or kidney organogenesis [17,18]. In many cases, dynamic E-cadherin expression takes place in the context of an Epithelial-to-Mesenchymal Transition (EMT), a developmental process characterized by loss of cell polarity and adhesion (characteristic of epithelial cells), with vast morphological changes and acquisition of cell motility (mesenchymal state) [18]. EMT is to a large extent coordinated by so-called classical EMT transcription factors: Snail, Slug, Twist, ZEB1, and ZEB2. These are well-characterized transcriptional repressors, all of which can directly bind to and repress E-cadherin promoter and other cell-cell adhesion and epithelial genes [22,23].

Less is known about the physiological roles of the other previously referred classical cadherins. N-cadherin has a broader expression profile, present in nervous, fibrous, and musculoskeletal tissues [24–27], and is considered a cell-cell adhesion molecule expressed by mesenchymal cells. Similar to E-cadherin, N-cadherin is critical in cell attachment [28], differentiation into specialized tissues [24–27], and influence signaling in various cellular processes, such as cell proliferation and apoptosis [29]. Although N- and E-cadherin share structural and functional characteristics, they usually show a mutually exclusive

expression pattern during embryonic morphogenesis [30]. Notably, N-cadherin adhesion properties appear to be strongly tissue-specific. It mediates strong cell-cell adhesion in cardiomyocytes, but it is also expressed in migratory fibroblastic cells [24,31]. Concerning P-cadherin expression in human tissues, it partially overlaps with E-cadherin expression, possibly reflecting partial redundancy. The two proteins have high homology and differ mainly in their extracellular portions. P-cadherin is co-expressed with E-cadherin in embryonic stem cells and several adult epithelial tissues, including the breast, prostate, several organs of the digestive tract and urinary tract, lung, and endometrium (reviewed in [19,32]). Concerning R-cadherin in normal tissues, it is found to play an important role in brain segmentation and neuronal outgrowth and in lens, muscle, and kidney development.

2.1. Epithelial Tissues

Epithelia are robust tissues formed by sheets of cells organized as mono or multilayers that serve as effective barriers that support the structure and regulate functionally diverse organs, such as lung, gut, kidney, and epidermis (reviewed by [33]). Epithelia characteristically show strong cell-cell adhesion mediated by specialized adhesive sites, mainly tight junctions (TJ), adherens junctions (AJ), and desmosomes.

E-cadherin is expressed in all mammalian epithelia, being mainly co-expressed and located at the cell membrane and organizing the adherens junctions. Adherens junctions are located between the apical and basolateral membrane domains of epithelial cells and are linked to a circumferential actomyosin belt, a dynamic structure that participates in epithelial sheet remodeling and regulates epithelial tissue integrity [34–36]. Moreover, the interplay between E-cadherin complexes and the actin cytoskeleton enables resistance to cell deformation, granted by cell-cell adhesion; or otherwise triggers cellular remodeling, which tolerates epithelial plasticity [37,38].

Although a key component in epithelial polarization, E-cadherin is crucial to preserve epithelial tissue integrity and homeostasis through the stabilization of cell-cell interfaces and plays an important role in epithelial cell proliferation and migration control [39–41]. Accordingly, disruption of epithelial polarity has been shown to cause a wide range of human diseases [15,42–44]. Likewise, many studies have pointed to E-cadherin as a central protein in human epithelial cancers [45,46]. The loss of E-cadherin-mediated cell-cell adhesion is a prerequisite for tumor cell invasion. Reestablishing E-cadherin function in cultured tumor cells has been shown to reverse an invasive mesenchymal phenotype to a more benign and epithelial phenotype [45,47,48]. Furthermore, in several human cancer types, in parallel to E-cadherin loss, the de novo expression of mesenchymal cadherins, such as N-cadherin and cadherin-11, is observed during tumor progression [49–51]. These observations led to the concept of "cadherin switch" in cancer, parallel to what is observed during delamination and migration of epithelial cells during embryonic development.

P-cadherin expression is restricted to the basal layers of stratified epithelial tissues, including breast, skin, prostate, and lung [8,52,53]. When expressed, P-cadherin appears crucial for normal epithelial architecture [54] and cellular migration [55]. Furthermore, in addition to its role as an adhesion molecule, P-cadherin has been hypothesized to play an important role in cell differentiation and proliferation [56,57]. In the mouse mammary gland, P-cadherin knockout has been associated with abnormal mammopoiesis and increased risk for the development of preneoplastic lesions [58]. *CDH3*/P-cadherin mutations have been associated with abnormal development syndromes, including hypotrichosis with juvenile macular dystrophy (HJMD) and ectodermal dysplasia, ectrodactyly and macular dystrophy (EEM syndrome) [59,60].

2.2. Central Nervous System (CNS)

Cadherins are also highly expressed in the nervous system promoting cell-cell interactions within neural networks and dynamically contributing to neural development and function [61–65].

Various cadherins are widely expressed in neural tissues, including N-cadherin, cadherin-11, cadherin-6, cadherin-8, or M-cadherin [61,63,64,66]. Initially, E-cadherin is also expressed in embryonic ectoderm, being replaced by N-cadherin after neural induction, which leads to the formation of the neural plate [67]. As cell differentiation proceeds in the neural tube, cadherin expression becomes restricted to specific brain subdivisions. For instance, in the mouse developing brain, R-cadherin expression is distributed throughout the telencephalon and hindbrain, while cadherin-8 expression is restricted to the cerebellum and ventral thalamus [64].

In the adult brain, cadherins' expression decreases and often remain localized to synapses or perisynaptic areas [65,68]. However, past observations suggest that cadherins are involved in various aspects of neural development and function, including organization of neuroepithelial layers [9,67], regulation of neuronal migration [69], complex functional subdivision, and early compartmentalization [70] by modulating region-specific adhesiveness. Furthermore, an important role in synaptic function and plasticity has been pointed out for cadherin-catenin complexes [65,68]. In particular, N-cadherin has been shown to play a similar role in neural tissues as E-cadherin in epithelia. This protein grants cell polarity and the maintenance, proliferation, and differentiation of neural progenitor cells [71].

In normal glial cells, N-cadherin is also thought to be the key cadherin. Although with scarce literature, it was identified in oligodendrocytes and their precursors [72], as well as in astrocytes [73,74]. Neural phenotypes associated with cadherin loss of function include defects in neural tube formation and cortical organization, cognitive and synaptic dysfunction, failure of sensory neurogenesis, CNS malformations, and craniofacial development. In addition, cadherin-catenin complexes are involved in CNS developmental steps and synapse formation and functions. It is thus not surprising that defects and alterations in the cadherin-catenin complex impair high-order neural functions. In particular, it has been associated with addiction-related phenotypes, schizophrenia, and bipolar disorder [75–77].

3. Cadherins Expression in Neoplastic Disease

3.1. Systemic Cancer

During oncogenesis in epithelial tissues, organized cell-cell adhesion and normal cell polarity are disrupted by genetic, epigenetic, and microenvironmental changes, leading to abnormal signaling, loss of contact inhibition, altered stromal interactions, and cell migration (reviewed in [19]). E-cadherin was investigated in seminal studies regarding its role in carcinomas, which are tumors of epithelial origin. Since then, various studies have proposed the CDH1/E-cadherin coding gene as a tumor suppressor gene by enabling complex mechanisms to promote tissue organization and apoptosis blockade [5,49,78]. These mechanisms include both biophysical cell-cell adhesion processes and intracellular signaling coupled to inhibition of proto-oncogenic molecules, such as βcatenin and epidermal growth factor receptor (EGFR) [18,79]. Moreover, suppression of E-cadherin function has been linked to increased cell migration and invasion and induction of a mesenchymal cellular morphology [80]. E-cadherin loss was demonstrated in a variety of cancers, including lobular breast carcinomas [78], gastric adenocarcinoma [81], hepatocellular carcinoma [82], melanoma [83], squamous cell carcinomas of the skin [84], as well as esophagus [85], and head and neck carcinomas [86,87]. More recently, data from other cancers have questioned the loss of E-cadherin expression as a ubiquitous marker of disease aggressiveness [88,89]. In later stages, invasive and poorly differentiated breast and ovarian carcinomas, for example, still express E-cadherin, which marks the existence of a mesenchymal to epithelial transition during tumor progression or a hybrid or mixed epithelial-mesenchymal phenotype [90,91]. Since the initial reports, the role of other cadherins in cancer, including

its actions as synergists or antagonists with E-cadherin, has been sought. Importantly, the structural diversity of cadherins and, particularly, their tissue-specific expression profiles and actions were indicators that cadherins' role in oncogenesis was most likely heterogeneous. Initial studies in carcinomas have labeled N-cadherin as a "mesenchymal" biomarker; and tied it to the end-stage of a pathological, cancer-related EMT [92]. Indeed, cadherins frequently exhibit a homotypic binding pattern across cells. For N-cadherin, frequently expressed in stromal and endothelial cells, this leads to epithelial heterotypic cell-cell adhesion, facilitating local invasion and dissemination of carcinoma cells [93]. Various studies have elicited the role of N-cadherin in tumor progression [94]; however, in others, N-cadherin loss has been linked to a worse prognosis [95]. As for E-cadherin and N-cadherin, P-cadherin cancer-related function seems to be context-dependent [96,97]. In breast cancer, expression of P-cadherin is characteristically observed in tumors with high proliferative rates and decreased cell differentiation, strongly associated with poor patient survival [57,98,99]. By contrast, in colon carcinomas, P-cadherin is strongly expressed in well-differentiated, while mostly absent in poorly differentiated colon tumors [100]. P-cadherin overexpression with E-cadherin suppression and N-cadherin induction are considered components of the cadherin switch found in cancer-associated EMT responsible for tumor differentiation and progression [101]. Nevertheless, rising evidence points to the importance of considering the cancer-dependent role of these proteins, as much as the putative interactions between them. In a model of breast cancer, co-expression of P- and E-cadherin was associated with aggressive high-grade breast carcinomas by P-cadherin-mediated disruption of functional cadherin-catenin complexes, thereby obstructing the E-cadherin tumor-suppressive role [97,102–104]. Considering R-cadherin impact on tumorigenesis, its role is still controversial. Conflicting data points R-cadherin as either promoting tumor progression [105] or inhibiting it [106,107].

3.2. CNS Tumors

As mentioned before, N-cadherin is the most commonly expressed cadherin in the CNS. E-cadherin expression, in particular, is rare and localized to arachnoid cells, choroid plexus, and anterior pituitary [108]. Peripheral nervous system cells, including Schwann cells, were also found to express E-cadherin. This protein plays an important role in cell adhesion and maintenance of peripheral nervous system architecture [109–111]. Consequently, the role of E-cadherin expression in tumors derived from these locations has been studied and described in meningiomas, choroid plexus papilloma, schwannomas, and pituitary adenomas [108,112–116].

3.2.1. Pituitary Adenomas

Pituitary adenomas are epithelial tumors with neuroendocrine differentiation, immunohistochemically subdivided according to the presence of pituitary hormones and transcription factors. Variable E-cadherin and N-cadherin staining has been described in these tumors [114,117–119]. In particular, loss of E-cadherin expression has been linked to increased tumor dimensions and invasive behavior in growth hormone and prolactin-producing pituitary adenomas [114,120–122]. In addition, response to pharmacological treatment with somatostatin analog was worse for somatotroph adenoma without E-cadherin expression [114]. In a surgical series of 52 patients, E-cadherin loss and N-cadherin expression accompanied morphological changes suggestive of an EMT-like process, unique to a subset of invasive pituitary adenomas [123].

3.2.2. Meningiomas

Various studies have found E-cadherin to be expressed in diverse subtypes of meningiomas, tumors derived from arachnoid cap cells [108,112,116]. Furthermore, E-cadherin downregulation has been described as one of the main molecular events responsible for meningioma development, and alterations within the CDH1 gene have been found in this neoplastic disease, namely loss of heterozygosity (LOH) and genomic instability [112,124].

Loss of E-cadherin expression has been associated with tumor proliferation, invasiveness, and dedifferentiation [116,125,126]. In addition, E-cadherin expression is less frequently observed in atypical (WHO grade II) meningiomas and is commonly absent in anaplastic meningiomas (WHO grade III), which are considered malignant [124,126]. Moreover, one study found that E-cadherin loss of expression was a significant predictor of tumor recurrence in meningioma patients [126].

In contrast to meningiomas and pituitary adenomas, the role of classical cadherins in other CNS tumors remains to be clarified, as published data remain limited and conflictive. For instance, leptomeningeal dissemination has been associated with increased N-cadherin expression in medulloblastoma [127], while lower expression levels were identified in disseminated neuroblastoma [95] and ependymoma [128]. Importantly, a recent study showed frequent positivity for N-cadherin in ependymoma and identified increased N-cadherin levels as a predictor of earlier tumor recurrence, arguing a contrary role for N-cadherin as a marker of worse prognosis in ependymoma [129]. Studies on E-cadherin expression show that this adhesion protein is mostly absent in neuro-epithelial tumors, such as ependymoma and medulloblastoma [113,125,127].

3.2.3. Gliomas

The expression patterns of classical cadherins in gliomas have been evaluated by several, yet contradicting, studies. Gliomas characteristically diffusely infiltrate the underlying cerebral parenchyma, which has normal N-cadherin expression. This behavior reflects a higher tendency toward tumor invasion and migration, which rely on cell-cell adhesion mechanisms.

N-cadherin expression in gliomas has been described in various patient cohorts, with approximately 60–80% of positive cases in glioblastoma series [130,131]. However, these studies are discordant regarding the correlation between N-cadherin immunoreactivity with patient prognosis. In 1995, Shinoura et al. studied the differences in N-cadherin expression in the normal brain parenchyma and different gliomas, including pilocytic astrocytomas, low-grade oligodendrogliomas or astrocytomas, anaplastic astrocytomas, and glioblastomas. Although mRNA levels for N-cadherin were significantly higher in glioblastomas, protein expression was similar among high-grade and low-grade gliomas and normal brains. Moreover, no consistent association between invasiveness capacity and N-cadherin expression was found [132]. As for Shinoura et al., other studies have found discrepant results between N-cadherin mRNA and protein expression levels [133,134]. These results point to the importance of protein stability in the tumor microenvironment. Indeed, a study showed that N-cadherin cleavage by ADAM-10 occurred at a significantly higher rate in glioblastoma cells than in normal brain [135], and it has been further suggested that N-cadherin cleavage is a prerequisite for glioblastoma cell migration [5,135].

Other studies have supported a protective role for N-cadherin in gliomas. In glioma models, N-cadherin down-regulation has been associated with altered cell polarization and abnormal motile behavior [136], with a significant increase in tumor cell migration and invasive capacity [136,137]. In surgical specimens, expression levels evaluation pointed to a decrease in N-cadherin immunoreactivity upon glioblastoma recurrence [138,139] and association to tumor cerebrospinal fluid (CSF) dissemination [139]. In line with this, another report described how even redistribution of N-cadherin at the cell membrane is induced by cleaving its intracellular anchorage to the cytoskeleton by ROBO1, promoting invasiveness of glioblastoma cancer stem cells [140].

Nevertheless, contradictory results have questioned the role above of N-cadherin in glioma genesis. N-cadherin protein expression levels were also shown to increase according to pathological glioma grade [130,141] and correlate with Ki-67 labeling index [130], suggesting a role for cell adhesion signaling in tumor cell proliferation and dedifferentiation. Importantly, a trend towards decreased survival with increased expression of N-cadherin has also been described in different cohorts [131,141]. In a very recent publication, Gritsenko et al. studied the mechanisms of brain infiltration by glioma cells. They proposed an

AJ-mediated mechanism for glioma migration and progression, dependent on N-cadherin and p120-catenin complex [142]. Similarly, Osuka et al. showed an N-cadherin expression increase among the population of radioresistant glioma stem cells (GSC), with increased stemness and reduced proliferation [143].

E-cadherin expression has been more commonly described as scarce or absent in gliomas [120,125,131,141] while contradicting results regarding its role in disease progression have been hypothesized. Motta et al. analyzed E-cadherin mRNA levels in 81 neuroepithelial tumors, including 62 astrocytic tumors and those from WHO grade I to IV. Low-grade astrocytomas (grades I-II) showed higher mRNA levels than did high-grade astrocytomas [144]. Likewise, Bar et al. reported E-cadherin staining to be more commonly positive in grade II (43%) over grade IV gliomas (23%) [145]. These results suggest that a decrease in E-cadherin gene expression underlies astrocytoma progression. Moreover, in a series of low-grade gliomas, CDH1 promoter methylation status was found to be frequent (65% astrocytomas, 66% oligodendrogliomas, and 57% oligoastrocytomas) and hypermethylation status associated with shorter progression-free survival [146]. In the same study, negative immunoreactivity for E-cadherin correlated with shorter patient progression-free and overall survival [146]. Despite these results, the assumption that E-cadherin plays the same protective role in gliomas as in most systemic cancers remains highly dubious. Opposing data documented E-cadherin expression to be higher in high-grade gliomas than in low-grade gliomas [147]. Furthermore, in particular subtypes of glioblastoma, with epithelial and pseudo-epithelial differentiation and high E-cadherin positivity, E-cadherin expression correlated with a worse prognosis [148,149]. In glioma cell models, E-cadherin expression was confirmed to be a rare event but to significantly influence in vitro growth and invasion capacity [148]. All this information is summarized in Table 1.

Table 1. Summary of cadherin's expression and prognosis in different published patient glioma series. NA (not assessed).

Author/Year	Cadherin and Method	Result	Prognosis
Howng et al. 2002 [120]	E-cadherin expression by RT-PCR in 16 astrocytomas and 29 other SNC tumors	Ecad staining in 2/16	NA
Schwechheimer et al. 1998 [125]	E-cadherin Immunohistochemistry of 42 gliomas (7 grade I astrocytoma, 6 grade II astrocytima, 5 grade II oligodendroglioma, 14 grade III astrocytoma, 8 GBM) and other 105 SNC tumors	No Ecad staining in gliomas	NA
Utsuki et al. 2004 [127]	E- and N-cadherin Immunohistochemistry of 45 gliomas (18 GBM, 16 grade III anaplastic astrocytomas, 11 grade II diffuse gliomas).	No Ecad staining Ncad staining in GBM (81%) and anaplastic astrocytoma (31%)-Ncad staining increases with WHO grade.	Ncad staining associated with a worse prognosis
Noh et al. 2017 [131]	E- and N-cadherin Immunohistochemistry of 92 gliomas	Ecad expression in 8.7% Ncad expression in 88.0% No significant difference in OS, PFS increased in low Ncad expression.	Tendency for worse prognosis with Ncad expression
Shinoura et al. 1995 [132]	N-cadherin mRNA and protein level in 21 gliomas (one pilocytic astrocytoma, 4 grade II astrocytomas, 3 grade III anaplastic astrocytoma, 9 GBM, 2 mixed gliomas, 2 grade II oligodendroglioma)	Ncad expression equal (protein) to higher (mRNA) in GBM	No prognosis
Asano et al. 1997 [138]	N-cadherin Immunohistochemistry of 22 astrocytomas (13 GBM and 9 anaplastic grade III astrocytoma)	No Ecad expression Ncad expression in all primary tumors. Ncad loss associated with tumor dissemination.	Ncad staining better prognosis.

Table 1. *Cont.*

Author/Year	Cadherin and Method	Result	Prognosis
Wu et al. 2013 [141]	E- and N-cadherin Immunohistochemistry of 40 brainstem gliomas (30 low grade and 10 high-grade) and RT-PCR of 18 brainstem gliomas (10 low grade and 8 high grade)	No Ecad staining, weak mRNA level. N-cad expression increased with WHO grade and showed a trend toward shorter survival.	Ncad expression associated with a worse prognosis
Motta et al. 2008 [144]	E-cadherin RT-PCR in 62 astrocytomas (21 grade I astrocytomas, 10 grade II astrocytomas, 10 grade III astrocytomas, and 21 GBM) and 19 other SNC tumors	Ecad expression is higher in low-grade astrocytomas than high-grade astrocytomas. Ecad expression decreases with WHO grade.	Ecad expression associated with a better prognosis
Bar et al. 2014 [145]	E-cadherin Immunohistochemistry of 92 gliomas (23 grade I pilocytic astrocytoma, 23 grade II astrocytoma, 7 grade II oligodendroglioma, and 39 GBM.	Ecad staining in 28.8% of gliomas, no differences with WHO grade.	No prognosis
D'Urso et al. 2011 [146]	E-cadherin Ecad gene (CDH1) promoter methylation and E-cad expression by methylation-specific polymerase chain reaction (MSP) and immunohistochemistry in 84 low-grade gliomas (43 diffuse astrocytomas, 27 oligodendrogliomas, and 14 oligoastrocytomas)	CDH1 promoter hypermethylation in 65% of astrocytomas, 66% oligodendrogliomas, and 57% oligoastrocytomas correlated with a worse prognosis. Ecad expression positive in 15/43 astrocytomas, 9/27 oligodendrogliomas, and 6/14 oligoastrocytomas. Loss of immunoreactivity for E-cadherin correlated with worse survival	Loss of Ecad expression worse prognosis
Lewis-Tuffin et al. 2010 [148]	E-cadherin Immunohistochemistry of TMAs of 83 cases of GBM or anaplastic astrocytoma and 31 gliomas and immunohistochemistry of 27 GBM with epithelial/ pseudo-epithelial differentiation	No Ecad expression in TMA Ecad staining in 33% of GBM with epithelial/pseudo-epithelial differentiation. Worse overall survival in patients with Ecad expression.	Ecad staining associated with a worse prognosis.
Rodriguez et al. 2008 [149]	E-cadherin Immunohistochemistry of 58 GBM with adenoid, epithelioid, or true epithelial features	Ecad staining in 82% of GBM.	NA
Darweesh et al. 2016 [150]	N-cadherin Immunohistochemistry of 60 GBM	Ncad expression in 88.3% cases. Ncad expression associated with tumor histological variant-more common in cases of Gliosarcoma than in glioblastoma with oligodendroglioma component.	NA

Almost inexistent literature can be found for the other two classical cadherins in glioma genesis. Nonetheless, contradicting data for R-cadherin has been described. On the one hand, R-cadherin increased expression has been shown at the cell-cell junctions after PDGF-B-induction in an experimental model of high-grade oligodendrogliomas [151], thus suggesting a role in glioma genesis. On the other side, it has been demonstrated that it is upregulated after autophagy induction, promoting a more epithelial and less aggressive phenotype of glioblastoma cell lines [152].

4. EMT and Cadherin Switch in Gliomas

Glioma capacity to grow, invade and resist conventional therapy, akin to systemic cancer, relies on EMT-like cellular programs [153]. However, the significance of the EMT and MET (mesenchymal to epithelial transition) programs in cancer progression and metastasis remains to be fully elucidated. In particular, how the concept of EMT applies to glioblastoma and other factors tumors with non-epithelial origin.

In 2010, transcriptomic analysis of glioblastoma established the presence of a molecular classification into four subtypes: proneural, neural, classical, and mesenchymal [154]. Since then, the mesenchymal subtype of glioblastoma has been thoroughly investigated and suggested to associate with an invasive phenotype, increased aggressiveness, and robust treatment resistance [153,155–157]. The mesenchymal phenotype in glioblastoma has, in addition, been correlated with worse overall survival [154]. Moreover, the mesenchymal transformation has been documented in glioblastoma upon tumor recurrence [155,156], as well as a response to various insults, including temozolomide [158] and radiation treatments [159]. Perhaps the strongest evidence for an EMT-like process in glioblastoma is the activity of classical EMT transcription factors and other well-described EMT-promoting pathways, such as ZEB1/ZEB2 [140,160–163], TWIST1 [164–166], WNT/β-catenin pathway [167,168] and SNAI2/SLUG [159,169] (Figure 1). An EMT-like program in gliomas is thus increasingly recognized and correlated to the phenomenon of glioblastoma progression and invasion. However, the canonical EMT-related E- to N-cadherin switch is unlikely to be an essential process. Increasing evidence suggests that cadherins may not be restrictive to have a specific role in tumorigenesis but instead can work as tumor suppressors or promotors depending on the tissue and tumor-associated context [170–173].

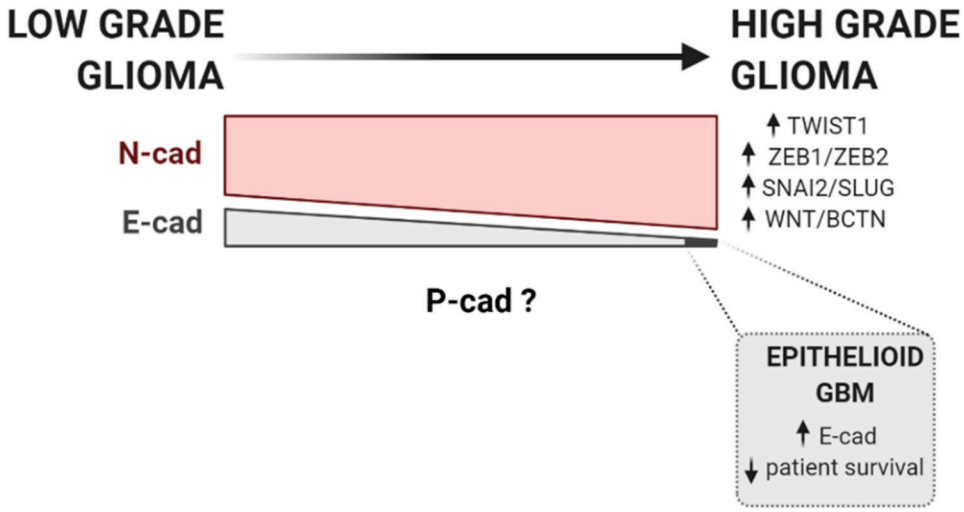

Figure 1. Cadherins and EMT factors expression during glioma progression. The progression of low-grade to high-grade glioma is commonly accompanied by an increase in the activity of classical EMT transcription factors and other well-described EMT-promoting pathways, such as ZEB1/ZEB2 [140,160,161], TWIST1 [164,165], WNT/β-catenin pathway [167], and SNAI2/SLUG [159,169]. According to current data available, N-cadherin is generally expressed in low-grade glioma, but its expression has been described to be increased in high-grade gliomas [130,141]. On the contrary, E-cadherin is fairly absent in gliomas. Still, its expression is more frequent in low-grade gliomas than in glioblastoma [144,145]. Notwithstanding, in aggressive glioblastoma subtypes, with epithelial and pseudo-epithelial differentiation, E-cadherin is found to be overexpressed [148]. Upward row represents increased expression, whereas downward row indicates decreased patient survival rate.

Taken together, published data point for a broad N-cadherin expression in glioblastomas, with a likely increase from low grade to high-grade gliomas [130,141]. The tumorigenic result of such expression is disputed, as it spans from decreased invasiveness and dissemination capacity [136,137,139] to a worse prognosis with decreased overall

survival in clinical series [131,141] (Figure 1). Data on E-cadherin, whose expression is, on the contrary, fairly absent in gliomas, proposes E-cadherin loss as a potential step in glioma genesis, as the expression is more frequent in low-grade gliomas than in glioblastoma [144,145]. Notwithstanding, important contradictory data in apparent aggressive glioblastoma subtypes, with epithelial and pseudo-epithelial differentiation and high E-cadherin expression, questions such a proposed benign role for E-cadherin in gliomas [148] (Figure 1). It is nevertheless misleading to simply interpret these results in the context of a systemic EMT. They emerge from a collection of small sampled patient series [120,125,130,132,138,141,145,146,149], in which E-cadherin expression is substantially lower and N-cadherin expression considerably higher than in published carcinomas series [57,174–177]. Importantly, no key reciprocal or concomitant down and upregulation for these proteins was explored or suggested, as the role of cadherin co-expression was not explored. Moreover, data on P-cadherin expression in glioblastoma is at the moment inexistent Figure 1.

Caution is therefore mandatory when interpreting results for cadherin expression and its role as EMT biomarkers in glioblastoma, as proof of a cadherin switching process remains elusive in glioblastoma and cadherin expression contribution to glioma progression is still unclear.

5. Conclusions

Cadherins are key proteins in normal development, and tumor pathology and data on cadherin expression in gliomas point to a distinct pattern of expression when compared to classical epithelial cancers, in which the prognostic significance of each cadherin remains elusive. In order to understand how cadherin expression reflects EMT and differentiation status in gliomas, as well as propensity to invade and migrate, it is crucial to develop large-scale studies in low-grade to high-grade gliomas, with the concomitant evaluation of both epithelial and mesenchymal cadherins.

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LABORATORY INVESTIGATION

PD-L1 tumor expression is associated with poor prognosis and systemic immunosuppression in glioblastoma

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Abstract

Purpose Glioblastoma is the most common primary malignant brain tumor in the adult, whose grim prognosis largely relates to the absence of effective treatment targets. Given its success in other cancers, immunotherapy has been trialed in glioblastoma and failed to demonstrate the expected benefit. Importantly, these disappointing results highlight the importance of understanding the unique and transforming biology of glioblastoma and its microenvironment. Our goal was to evaluate and characterize the expression of PD-L1 through immunohistochemistry in a large glioblastoma cohort. We further studied PD-L1 expression-associated prognosis and its correlation to systemic and neuropathological parameters.

Methods A series of 352 glioblastoma specimens (313 initial resection, 39 matched recurrences) was collected, with a detailed characterization of tumor neuropathological characteristics, including the presence, density and location of tumor infiltrating lymphocytes (TIL). Two hematological markers, absolute lymphocyte count and neutrophil–lymphocyte ratio (NLR), were used to analyze and correlate with systemic inflammation and immunosuppression. Immunohistochemistry was performed to evaluate PD-L1 expression.

Results Membranous PD-L1 expression was identified in 31% (98/313) of newly diagnosed and 46% (18/39) of matched recurrent tumors. TIL were found in 26% (82/313) of primary tumors and both density and location were found to be significantly associated with PD-L1 expression ($p < 0.001$). Interestingly, PD-L1 expressing tumors had more frequently areas with sarcomatous differentiation ($p < 0.001$) and were significantly associated with lower lymphocyte count ($p = 0.018$) and higher NLR ratio ($p = 0.004$) upon diagnosis. Importantly, PD-L1 expression was an independent poor prognostic marker in our cohort.

Conclusion Taken together, our data points to a putative role for PD-L1 expression in glioblastoma biology, which correlates to poor patient overall survival, as well as with a general systemic inflammatory status and immunosuppression.

Keywords Glioblastoma · PD-L1 · Tumor infiltrating lymphocytes (TIL) · Neutrophil–lymphocyte ratio (NLR) · Lymphocytopenia

Introduction

Glioblastoma is the most aggressive form of gliomas, a group of primary tumors arising from glia or their precursors within the central nervous system. Despite their low incidence, these highly aggressive tumors encase a disproportionate rate of cancer-related mortality, with a 5-year overall survival of only 6.8% [1, 2]. The standard of care remains surgical resection followed by radiotherapy and chemotherapy, usually temozolomide [2]. Glioblastoma represents one of the biggest therapeutic quests in current oncology.

The development of effective treatments for glioblastoma is challenged by its intracranial location, rapid progression, infiltrative growth and extensive intratumoral heterogeneity. In addition, glioblastoma is one of the most immunosuppressive solid tumors [3], capable of inducing early systemic T cell dysfunction and escape from immunosurveillance [4–6]. Indeed, glioblastoma patients frequently exhibit severe lymphocytopenia comparable to AIDS-level CD4 counts, which has been shown to portend a worse prognosis [7, 8]. In parallel to systemic immunosuppression, inflammation has been

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thoroughly related to cancer initiation and progression [9, 10]. Inflammatory parameters, such as neutrophil-to-lymphocyte ratio (NLR), have been shown to reflect prognosis in various tumors [11, 12]. These results have been validated in glioma cohorts and an elevated NLR in peripheral blood has been suggested to reflect a worse patient prognosis [13, 14].

Apart from the systemic inflammation and immunosuppression, glioblastoma induces a significant local immunosuppression. Mechanisms of immunosurveillance escape include tumor expression of PD-L1, a negative regulator of lymphocyte activation and so-called immune checkpoint, which interacts with PD-1 expressed by activated CD4 + or CD8 + T-lymphocytes [15]. Antibodies that block the immune checkpoints have demonstrated activity in a variety of solid tumors, and are now standard of care for instance in brain-metastasized melanoma [16]. Several biomarkers of response to immune-checkpoint inhibitors have been identified and disputed, such as mutational burden [17, 18], DNA-repair deficiency [19], and checkpoint-ligand expression [20].

Amid the successes of immunotherapy in several tumor types, results for glioblastoma unfortunately suggest this tumor as an epitome of resistance to immunotherapy. Results for immune-checkpoint inhibition in newly diagnosed glioblastomas failed to show a benefit for the addition of nivolumab to radiation (CheckMate-498) [21] or radiation and temozolomide in MGMT-methylated patients (CheckMate-548) [22] when compared to radiation plus temozolomide. In recurrent glioblastoma, nivolumab failed to be superior to bevacizumab (CheckMate-143) [23]. In this particular clinical trial, only 7.8% of patients exhibited response to nivolumab compared to the 23.1% of patients with response to bevacizumab. The median duration of response to nivolumab, however, was a striking median of 11.1 months compared to 5.3 months for bevacizumab. These results suggest that patient selection might play a key role in the efficacy of PD-1 blockade for glioblastoma. Additional studies focused on identifying markers for glioblastoma response to immune-checkpoint inhibitors, pointing to a complex interaction by genomic and transcriptomic characteristics (such as *PTEN* and *BRAF* mutations) with the dynamic composition of the tumor microenvironment [24–26].

The notorious glioblastoma intratumoral heterogeneity urges for the identification of biomarkers that do not merely reflect its molecular characteristics, but mostly its dynamic evolution. We thus characterized PD-L1 expression in a large and well characterized glioblastoma patients' cohort, which includes paired recurrent samples, and aimed to establish patterns of expression associated to patient characteristics, systemic immunosuppression and inflammation, tumor molecular and neuropathological characteristics, including the presence of tumor infiltrating lymphocytes (TIL).

Materials and Methods

Human glioblastoma samples

A retrospective series of 313 patients who underwent biopsy or surgical resection of newly diagnosed glioblastoma (GBM), between 2010 and 2017, at the Neurosurgery Department, Hospital de Santo António—Centro Hospitalar Universitário do Porto, was included in the study. Formalin-fixed and paraffin-embedded (FFPE) tumor tissues were histologically analyzed for all primary tumors, as well as for matched local recurrences (39 patients). Clinical data, treatment decision and patient survival were extracted by chart review for all the cases included in the series. Initial full blood count data was collected and documented, data from 3 patients was excluded due to previous corticoid therapy. Lymphocytopenia was considered when lymphocyte count was inferior to 1000/μL. NLR (neutrophil-to-lymphocyte ratio) was calculated as absolute neutrophil count divided by absolute lymphocyte count. Initial MRI was also evaluated and imaging characteristics documented. Information regarding *IDH1 (R132H)* and *ATRX* mutations was available in 139 patients.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration. Moreover, it has been also approved by the ethics committee at Centro Hospitalar Universitário do Porto (2017-104 (093-DEFI/091-CES).

Neuropathological and tumor infiltrating lymphocyte evaluation

Neuropathological characteristics were analyzed in detail in hematoxylin and eosin staining from the initial tumor samples, including: necrosis presence and type (coagulative, pseudopalisade); thrombosed or glomeruloid vessels; mitosis; myxoid stroma; calcifications; papillary structures; oligodendroglial, gemistocytic, sarcomatous, epithelial and glial components. Tumor infiltrating lymphocyte (TIL) density was evaluated semi-quantitatively according to previously published data [27] into sparse, moderate or dense. In addition, TIL location was documented into 4 subsets: within glioblastoma tissue, perivascular region, perinecrotic region and brain infiltration zone [28].

PDL1 immunohistochemistry

Immunohistochemical staining for PD-L1 was performed in 3 µm sections using a mouse monoclonal antibody (clone 22C3, Dako), previously tested [29]. The assay was carried out on an automated immunostaining system (Ventana Benchmark Ultra, using the OptiView DAB IDH Detection kit, according to manufacturer's instructions). Human placenta has been used as a positive control and performed in parallel with FFPE sections from the GBM series. Positivity was considered membranous staining ≥ 5% in tumor cells [20].

Statistical analysis

Statistical analyses were performed using the computer software package SPSS (15.0, IBM). Chi-square test was used to assess group differences as appropriate. Overall survival from diagnosis of glioblastoma to death or last follow-up was estimated using the Kaplan–Meier limit method. The log-rank test was used to assess group differences. A 2-tailed significance level (p value) of 0.05 was applied.

Results

Association between GBM patients' initial lymphocytopenia and NLR with progression-free and overall survival

The clinical, hematological and treatment characterization of the patients' cohort are described in Supplementary Table 1. Half of the patients were lymphocytopenic upon tumor diagnosis (n = 167, 53,4%), uniformly distributed irrespective of age, gender and functional status. The presence of lymphocytopenia at diagnosis was significantly associated with a shorter progression free survival and overall survival (log rank test, $p = 0.03$ and $p = 0.022$, respectively) (Fig. 1A, B). Concerning the NLR, the mean value in our cohort was 9.654. Interestingly, *IDH1* and *ATRX* mutations had a lower NLR mean of 3.953 and 7.18, respectively. Higher NLR was also significantly associated with a shorter time to tumor recurrence (log rank test, $p = 0.010$) and shorter overall survival (log rank test, $p = 0.020$) (Fig. 1C, D). Interestingly, a cut-off value of 6 for NLR was found to stratify patient overall survival in a significant way (log rank test 12 vs 8 months, $p = 0.011$).

Neuropathological evaluation and TIL infiltration

Relevant histologic features of primary tumors are summarized in Table 1. A glial component was observed in 98.1% of the tumors, being the most proliferative component in most tumors. A sarcomatous component was evident in 18.2% and an epithelial component was identified in 13.4%. Positivity for *IDH1* mutation was observed in 5% of patients and in 17% of patients for *ATRX* mutation.

A total of 82 (26.2%) tumors showed TIL of variable density. Sparse-to-moderate TIL were preponderant (91.4%), with a minority of cases showing dense infiltration (8.5%). Table 1 details semi-quantitative evaluation of TIL density. These were predominantly localized in perivascular areas (50%) and tumor infiltrative zones (30.4%) and rarely showed a peri-necrotic location (8.5%). A tendency for worse overall survival for TIL location in tumor periphery was identified (8 months vs 12 months in perivascular location and 18.5 months within the tumor, p = 0.086). Interestingly, TIL were significantly more frequent in tumors with a sarcomatous component (55.9% vs 19.3%, chi-square, $p < 0.001$). No differences in patients' survival was observed regarding TIL infiltration subgroups, including TIL presence, density or location.

PD-L1 expression in primary and recurrent glioblastoma

PD-L1 staining of variable extent was found in 31% (n = 98) of the tumor samples (Supplementary Table 2; Fig. 2). In the majority of the patients, a granular membranous staining was found in sparse tumor cells. PD-L1 positivity in over 20% of the tumor was found in about 40% of positive cases, from which 8% showed staining in more than half of the tumor. Contiguous infiltrated or preserved brain parenchyma was evaluated when present, and a faint diffuse staining was identified in a minority of the samples (< 10%).

Matched primary and recurrent samples were obtained in 39 cases. Curiously, PD-L1 expression was higher in recurrent cases, in up to 46% of the samples. The primary tumor was negative for PD-L1 in 29 cases and positive in 10 cases. The majority of negative cases remained negative (n = 19), while there was gain of PD-L1 expression in tumor recurrence for 10 cases (34%). Of the 10 primary PD-L1 positive tumors, 8 maintained positivity and 2 cases lost PD-L1 staining.

Association between PD-L1 expression and clinicopathological characteristics

Relevant associations between PD-L1 expression and clinicopathological characteristics are summarized in Table 2. Patient age, gender, tumor location or multifocality did not correlate to tumor PD-L1 expression. There was also no correlation between preoperative treatment with corticosteroids and the expression of this biomarker. However, lower lymphocyte count on peripheral blood significantly correlated with PD-L1 positivity (chi-square, $p = 0.018$), as did a higher

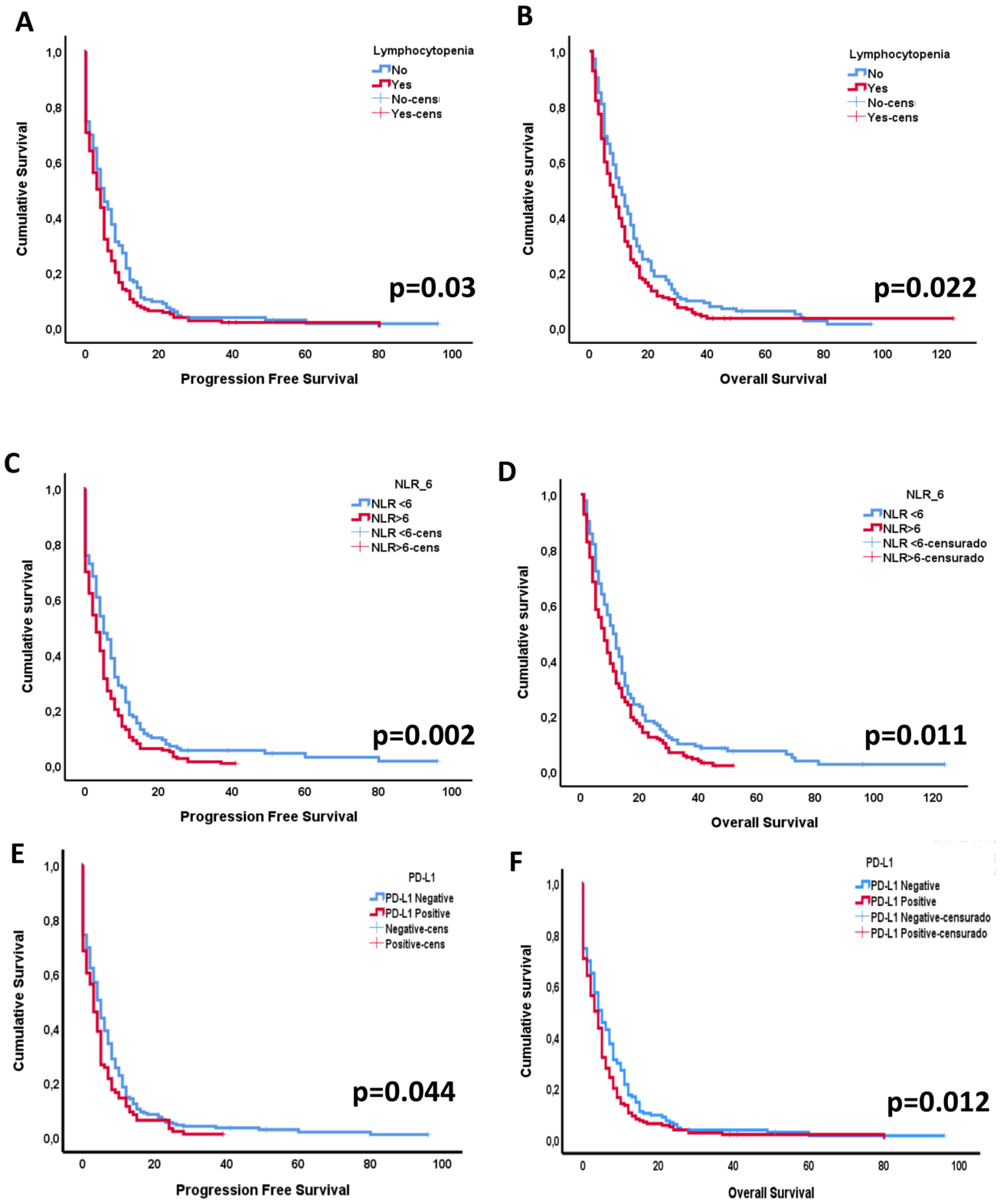

Fig. 1 Association between GBM patient's initial lymphocytopenia (**A**, **B**), NLR (**C**, **D**) and PD-L1 expression (**E**, **F**) with progression free survival and overall survival

Table 1 H&E evaluation of neuropathological features

			n	%	OS (months)	p-value
Tissue components		Glial	307/313	98.1	11	*0.046*
		Sarcomatous	57/313	18.21	8	
		Epithelial	42/313	13.4	10	
TIL	Density	Sparse	53/82	64.6	12	0.096
		Moderate	22/82	26.8	12	
		Dense	7/82	8.5	8	
		Very Dense	–			
TIL	Location	Perivascular	41/82	50	12	0.086
		Perinecrotic	7/82	8.5	12.5	
		Within tumor tissue	10/82	12.1	18.5	
		Tumor periphery	24/82	29.2	8	

Italics indicate the statistically significant of the p-values < 0.05

(> 6) initial NLR (chi-square, $p = 0.002$). PD-L1 expression was common in tumors with lymphocytic infiltrates (chi-square, $p < 0.001$), in particular with dense (72%) to moderate (83.3%) density. Moreover, PD-L1 expression was significantly associated with TIL location (chi-square, $p < 0.001$), most commonly found at tumor periphery (86.4%), compared with perivascular location (39%) or within the tumor tissue (1%). The majority of the tumors with sarcomatous features showed positivity for membranous PD-L1 (54.2%, chi-square, $p < 0.001$). Although not significant, tumors with *IDH* mutation were all negative for PD-L1 expression and less than 10% of patients with *ATRX* mutation showed PD-L1 expression.

Association between PD-L1 expression and patient's prognosis in glioblastoma

The established prognostic factors for GBM were validated in our cohort, including age (log rank test, $p = 0.001$), initial functional status (log rank test, $p = 0.001$), extent of resection (EOR) (log rank test, $p < 0.001$), complimentary treatment (log rank test, $p < 0.001$), IDH (log rank test, $p = 0.001$) and *ATRX* (log rank test, $p < 0.001$) mutations (Supplementary Table 3). Tumor membranous expression of PD-L1 in the primary tumor also significantly correlated to shorter time to recurrence (log rank test, $p = 0.044$) and worse overall survival (log rank test, $p = 0.012$) (Supplementary Table 3; Fig. 1E, F). Tumor PD-L1 expression showed a survival disadvantage for all treatment modalities, namely RT (log rank test, $p < 0.001$), sequential RT + QT (log rank test, $p = 0.025$), and Stupp protocol (log rank test, $p = 0.008$). Strikingly, in patients who did not received treatment following tumor biopsy or surgical resection, the survival disadvantage associated to PD-L1 expression was not observed (log rank test, $p = 0.418$). Although an increase in the percentage of positive cases was identified upon tumor recurrence, no significant correlation between immunostaining for PD-L1 in recurrent cases and patient prognosis was found. Importantly, on multivariate analysis, we identified PD-L1 expression in the primary tumor as an independent predictor of a worse prognosis (Table 3).

Discussion

PD-1/PD-L1 interactions are considered key to the immunological control of cancer and have been extensively explored in melanoma and epithelial cancers [30, 31]. Nevertheless, the rate of PD-L1-positive immunohistochemistry cases, correlation to clinical prognosis, and response to immunotherapy remains overall disputed [32–48]. Particularly in glioblastoma, immunohistochemistry has consistently shown higher PD-L1 expression in tumor specimens than in surrounding normal brain. Nevertheless, expression levels and subcellular distribution pattern varies greatly in the literature as a reflection of the variety of antibodies used for evaluation and PD-L1 positivity criteria (Supplementary Table 4). Published results more consistently correlate PD-L1 expression to glioma grade [32–34]and IDH-wild type genotype [32, 35–38]. Likewise, most published series suggest a negative role for PD-L1 expression in glioblastoma prognosis, although others reported no added value for PD-L1 expression in tumor prognosis [32–47] (Supplementary Table 4). Our main goal was to evaluate PD-L1 expression in a large series of glioblastoma patients, with over 300 primary tumors, while using 39 matched recurrences to document PD-L1 expression upon tumor recurrence. In our cohort, up to 31% of tumors showed PD-L1 staining, of which 20% had staining of more than 40% of tumor cells and 8.2% more than 50% of the tumor. A remarkable increase in staining in recurrent disease was identified, in up to 46% of the tumors. Furthermore, 36% of initial PD-L1 negative

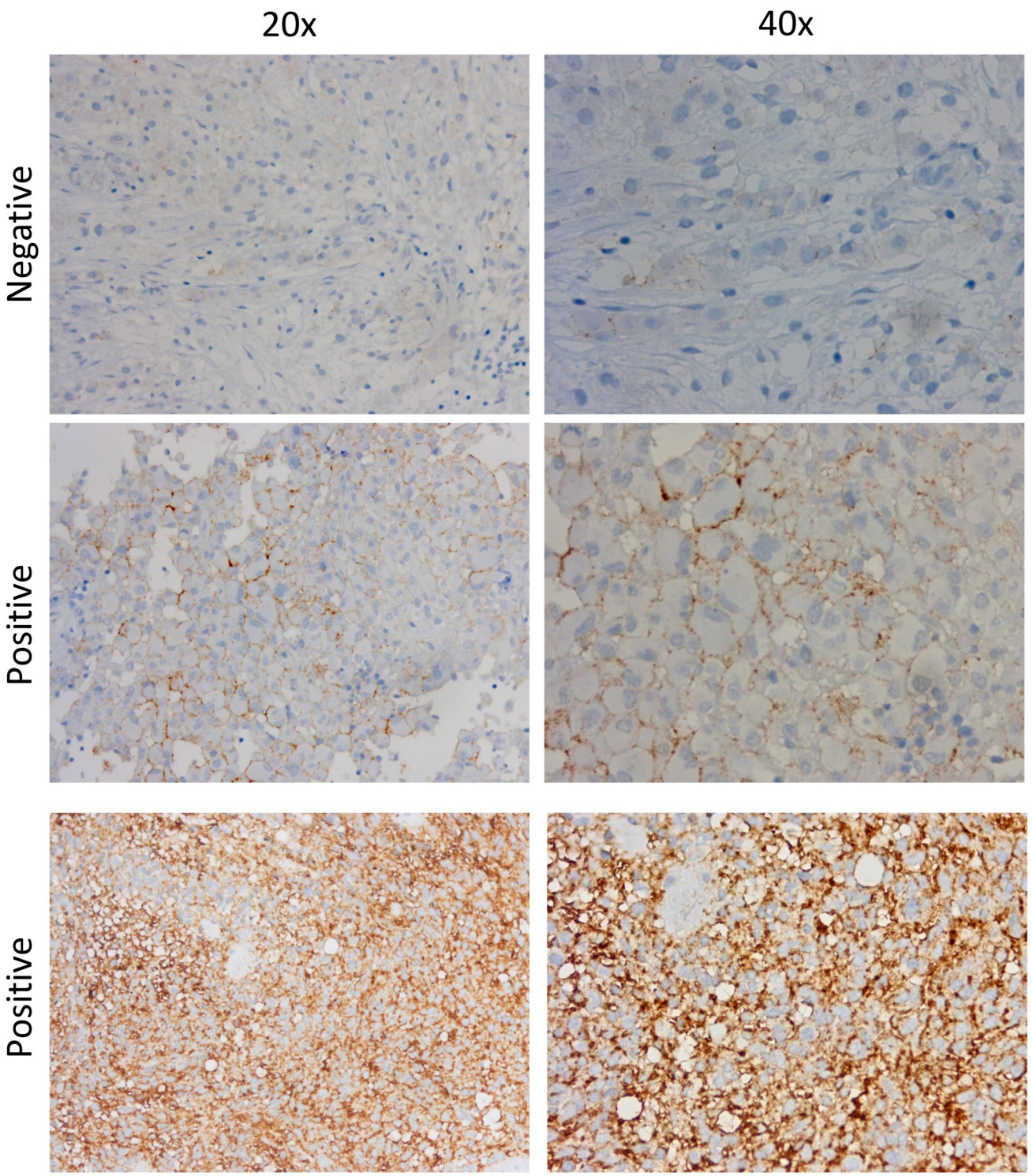

Fig. 2 Representative immunohistochemistry images for PD-L1 expression. ×20 and ×40 magnification

cases showed matched PD-L1 positive recurrent disease. These results support similar data suggesting a conjectural increase in PD-L1 expression as a mechanism associated to treatment resistance [48]. Furthermore, subgroup analysis by treatment modality curiously showed that, contrary to all treatment modalities, PD-L1 expression did not influence prognosis in treatment-naive patients. This data adds to the hypothesis that PD-L1-associated patient prognosis may mostly reflect treatment resistance mechanisms, as it foremost seems to impair the expected benefit of complementary

Table 2 Correlation between PD-L1 expression and clinicopathological characteristics

	PD-L1 expression		p-value
	Negative (n = 215)	Positive (n = 98)	
	n	n	
Lymph infiltrates/Inflammation			
No	177	54	*< 0.001*
Yes	38	44	
Lymph infiltrates/Inflammation density			
No	177	54	
Periphery	3	21	
Perivascular	21	19	*< 0.001*
Perinecrotic	5	3	
Tumor	9	1	
Lymphocytopenia			
No	110	36	*0.018*
Yes	105	62	
IDH			
No	89	43	0.69
Yes	7	0	
ATRX			
No	75	40	*0.032*
Yes	21	3	
Sarcomatous component			
Negative	192	62	*< 0.001*
Positive	23	36	
NLR			
NLR < 6	104	29	*0.002*
NLR > 6	111	69	

Italics indicate the statistically significant of the p-values < 0.05

Contingency tables and the chi-square test were used to estimate the relationship between PD-L1 staining patterns and the several parameters analyzed. The ANOVA test was used to evaluate the differences in tumor size, considering a confidence interval of 95%. Statistical analyses were performed by SPSS 15.0 software. *p* values less than 0.05 were considered statistically significant

treatments. We should, however, emphasize the retrospective nature of our study and further studies are needed in order to understand the dynamic expression of PD-L1 in the context of tumor local immunossupression, as well as its role in therapy resistance.

Nevertheless, our patients' survival analysis correlated PD-L1 positivity with a worse progression free and overall survival, further identifying PD-L1 expression as an independent prognosis factor in glioblastoma. This data concurs with similar series in the literature [49, 50] and, taken together, highlights the importance of the PD-1/PD-L1 axis in glioblastoma pathogenesis. In addition to the potential direct effect of an immunosupressive environment in patient prognosis; a putative relationship between the presence of tumor immune infiltrates, expression of immunosuppressive molecules and tumor subtype has recently gained rising importance. Taken together, recent studies propose disease-specific immune profiles, dependent on glioblastoma subtypes [51–53] with a consistent TIL-enrichment associated to a mesenchymal phenotype [25, 51, 53]. Indeed, PD-L1 positivity in our cohort was more frequent in IDH and ATRX wild-type tumors with areas of sarcomatous dedifferentiation. These results are in unison with reports correlating PD-L1 expression with the mesenchymal subtype in glioblastoma [28, 51, 54–56] as well as with data in systemic tumors, suggesting a relationship between tumor immune escape and the ability to undergo an EMT process. Tumor resistance to immune degradation may thus be, either or both, an inductor of a mesenchymal phenotype or perpetuated by the activation of EMT underlying pathways.

Other mechanisms have been proposed for glioblastoma local immunosuppressive environment, including cell surface factors, cytokine secretion/dysregulation, myeloid and lymphocyte dysfunction [57]. Initial case reports and small series have encouraged immunotherapy for glioblastoma [58–60], but results published to date on large clinical trials have failed to show a clinical efficacy [21, 23] and seemingly suggest that perhaps only a minority of

Table 3 Multivariate Cox proportional hazard analysis for classical prognostic factors, such as age, KPS, Extent of resection (EOR), Complimentary treatment and PD-L1

Variable	Evaluation	Multivariate Cox proportional hazard analysis			
		Progression free survival HR (95% CI)	*p* value	Overall survival HR (95% CI)	*p* value
Age	< 60 years	1		1	
	> 60 years	1.471 (1.065–2.032)	*0.019*	1.734 (1.256–2.393)	*0.001*
	< 70	1		1	
	> 70	0.742 (0.186–1.063)	0.103	0.648 (0.453–0.928)	*0.018*
Extent of ressection (EOR)	Biopsy	1		1	
	Partial	0.767 (0.385–1.525)	0.449	0.871 (0.436–1.737)	0.695
	Subtotal	0.754 (0.502–1.132)	0.173	0.636 (0.428–0.946)	*0.026*
	Total	0.511 (0.335–0.779)	*0.002*	0.400 (0.260–0.614)	*< 0.001*
Complimentary treatment	No	1		1	
	Yes	0.175 (0.112–0.276)	*< 0.001*	0.911 (0.212–0.458)	*< 0.001*
PD-L1	Negative	1		1	
	Positive	1.385 (0.995–1.927)	0.053	1.660 (1.190–2.315)	*0.003*

Italics indicate the statistically significant of the p-values < 0.05

This analysis allows the risk (hazard ratios and the corresponding 95% confidence interval, CI) to be predicted for disease-free and overall survival of breast cancer patients. A significance level of 5% was considered

glioblastoma patients benefit from these treatment strategies. Results on neoadjuvant treatment with pembrolizumab in recurrent glioblastoma demonstrated a significantly improved overall survival, suggesting that timing and association with other treatments should be explored [61]. In addition, a pivotal focus on T-cell dysfunction pairs the growing interest and applicability of immunotherapy to treat glioblastoma. Tumor immune infiltrates have been heterogeneously correlated to tumor prognosis in a variety of cancers [62, 63] This is partly explained by their diverse composition and functionality, as well as localization [64, 65]. Initial reports documented TIL presence in 28–60% of gliomas. Similar to other tumors, subsequent data on gliomas remains incongruous regarding TIL incidence, degree of infiltration and location (review by [66]). Absence of recent and consistent literature prevents an assertive correlation between glioma TIL and prognosis. In our cohort, TIL infiltrates were identified in a minority (26%) of tumors. These were more commonly sparse-to-moderate in density and located in perivascular areas or tumor periphery, rarely within tumor tissue. TIL presence and density did not correlate with patient prognosis. However, we identified a tendency for worse overall survival considering TIL location in tumor periphery. We interpret this result considering the so-called immune-excluded phenotype, in which T cell accumulate in tumor margins unable to invade the malignant tumor core [65]. Curiously, in our cohort, TIL location in tumor periphery was associated with PD-L1 expression in 21 of the 24 cases (87,5%) suggesting that a mechanism, perhaps the ability of tumor to induce T-cell dysfunction, may regulate T-cell location.

Apart from a profoundly local immunosuppressive environment, glioblastoma is capable of inducing a severe systemic immunosuppression, in particular with a tendency to cause systemic inflammation and severe systemic T-cell deficits [4, 5, 67]. Consequently, we explored two easily accessible and cost-effective markers of systemic inflammation and immunosuppression—initial lymphocyte count and NLR. We found that, early upon diagnosis, the majority of patients showed lymphocytopenia and high NLR, and that these two markers identified patients with a worse overall survival. In fact, NLR has consistently been described as an important factor for disease prognosis in glioblastoma and other systemic cancers [14]. However, how exactly a high NLR reflects tumor pathogenesis and, thus, prognosis is less clear: it may merely reflect a relative lymphocytopenia or neutrophilia; or, on the other hand, it may evidently identify

an abnormally coordinated inflammatory and immune response. Low T-cell counts have been previously conceived as a treatment-induced side effect, however, recent evidence has proposed T cell sequestration in the bone marrow as a mechanism for lymphopenia in glioblastoma, confirming that initial lymphopenia may well be a reflection of glioblastoma-associated systemic immunosuppression [4, 7, 68, 69].

In order to improve treatment efficacy, it is imperious to understand the intrinsic mechanisms associated to glioma immune responses, to characterize glioma subtype-defined immunophenotypes and to evaluate whether these differences lead to a clinically relevant patient stratification. Identification of predictive biomarkers of treatment response is mandatory. In a large cohort of patients, we emphasize the need to clarify the clinical relevance of lymphocytopenia, as well as TIL presence and location. Moreover, our data suggests that both lymphocytopenia and NLR warrant further evaluation as easily accessible and predictive biomarkers, as we speculate these alterations may reflect glioblastoma-induced immunossupression.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration. Moreover, it has been also approved by the ethics committee at Centro Hospitalar Universitário do Porto (2017-104 (093-DEFI/091-CES).

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Article

Cadherin expression profiles define glioblastoma differentiation and patient prognosis

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Abstract: Cadherins are cell-cell adhesion proteins, which have been strongly implicated in cancer invasion, dissemination and metastasis capacity, thus key players in the epithelial-to-mesenchymal transition (EMT) program. However, their role in glioblastoma (GBM), a primary central nervous system aggressive tumor, remains to be clarified. N-, E- and P- cadherin expression was analyzed on a large series of GBM, characterized with clinical, imaging and neuropathological parameters, as well as with patients' survival data. In addition, cadherins expression was studied in match-recurrent cases. Using TCGA data, cadherin expression profiles were also evaluated according to GBM transcription subtypes. N-cadherin expression was observed in 81.5% of GBM, followed by E-cadherin in 31% and P- cadherin in 20.8%. Upon tumor recurrence, P-cadherin was the only significantly upregulated cadherin compared with the primary tumor, being positive in 65.8% of the cases. Actually, P-cadherin gain was observed in 51.4% of matched primary-recurrent cases. Cadherins co-expression was also explored. Interestingly, E- and N-cadherin co-expression identified a GBM subgroup with frequent epithelial differentiation and a significant survival benefit. On the other hand, subgroups with P-cadherin expression carried the worse prognosis. P- and N-cadherin co-expression correlated with the presence of a mesenchymal phenotype. Expression of isolated P-cadherin or E- and P-cadherin co-expression were associated to imaging characteristics of aggressiveness, to highly heterogeneous tumors, as well as to worse patient survival.

Classical cadherins co-expression subgroups present consistent clinical, imaging, neuropathological and survival differences, which probably reflect different states of an EMT-like program in GBM.

Keywords: Glioblastoma; N-cadherin; E-cadherin; P-cadherin; EMT

Introduction

Cadherins are membranous calcium-dependent proteins crucial for cell-cell adhesion (1) and thus key players in a variety of cellular processes (2, 3). Most frequently, cadherins assemble into adhesive junctions between cells expressing the same cadherin subtype; however, their expression can also be highly heterogeneous, since cells can express more than one cadherin subtype (4, 5). Importantly, through their intracellular cytoplasmic tail, cadherins associate with numerous signaling proteins, participating in a dynamic process dependent on the cellular context and which culminates in the regulation of physiologic mechanisms, such as cell proliferation, differentiation, and migration (3, 6-9). Most likely, qualitative and quantitative differences in the expression of different cadherin subtypes confer tissue-specific characteristics and functional roles (2, 10).

Epithelial (E-), neuronal (N-), and placental (P-) cadherins are classical cadherins, the first members of the superfamily to be identified (*CDH1*, *CDH2*, *CDH3*, respectively). Apart from their important physiological roles (6, 8, 9), these proteins have been highly implicated in oncogenesis. Considering epithelial cancers, the loss of E-cadherin expression is considered a prerequisite for tumor cell invasion and dedifferentiation (11-14) and it frequently parallels with the *de novo* expression of mesenchymal cadherins, such as N-cadherin, a process called "cadherin switch", which is a marker of the epithelial to mesenchymal transition (EMT) molecular process (15-18). Nevertheless, evidence from other tumors has questioned this simplified model of cadherins expression in cancer (19-22). Tissue-specific expression profiles of cadherins suggest that, similarly to its cancer-related functions, cadherin expression subtypes must be context dependent, particularly when considering the added role brought by interactions between these proteins (23). Interestingly, current knowledge on the EMT process parallels with this concept of dynamic cadherins expression, with transitory and function-dependent regulation of gene expression, resulting in a complex non-binary program, frequently reaching a hybrid phenotype between epithelial and mesenchymal states (24). Importantly, the presence of various subclones with distinct epithelial, mesenchymal or intermediate/hybrid states within the same tumor has been observed (25), which strongly contributes to tumor heterogeneity.

In the Central Nervous System (CNS), the role of classical cadherins in gliomas remains to be clarified, as published data remain limited and conflictive (26-29). Gliomas are primary brain tumors notorious for their invasiveness, diffuse growth and treatment resistance (30, 31). Glioblastoma (GBM), the most frequent glioma, is a heterogeneous and aggressive tumor, with a median overall survival of 15 months (32, 33). In 2010, transcriptomic analysis of glioblastoma established the presence of a molecular classification into 4 subtypes: proneural, neural, classical and mesenchymal (34). Since then, the mesenchymal subtype of glioblastoma has been thoroughly investigated and suggested to associate with an invasive phenotype, increased aggressiveness and robust therapy resistance (35, 36). However, how the mesenchymal transcription subtype aligns with a phenotypic mesenchymal transition, or how the concept of EMT applies to GBM and to other tumors with non-epithelial origin, is still uncertain. In particular, the canonical EMT-related E- to N-cadherin switch is unlikely to be a central process in these neoplastic diseases and the assumption that N-cadherin expression marks for such a mesenchymal phenotype is likely farfetched. A recent review highlights how our current knowledge about cadherin expression in gliomas arises mostly from small sampled series, with significant contradicting results (29). For instance, N-cadherin expression has been associated with both: a protective role, with a decrease in tumor migration, invasiveness and dissemination (27, 37-39); as well as with a detrimental role, with a documented association between increased N-cadherin expression and high pathological glioma grade (40, 41), the presence of radioresistant glioma stem cells (42), and a significant decrease in patient survival (28, 41). Taken together, published results collectively point to a broad N-cadherin expression in gliomas, with a likely increase from low grade to high grade gliomas (41, 43). E-cadherin expression in gliomas is reduced and suggested to be more frequent in low grade gliomas than in GBM (44, 45). Interestingly, a recent paper studied, for the first time, P-cadherin expression on gliomas and demonstrated increased expression in high grade tumors and a correlation with a decreased overall survival in GBM (46). Moreover, P-cadherin expression was shown to induce an increased tumorigenic behavior, invasive capacity and stem-like characteristics in GBM cell lines (46).

Overall, conclusions of published results on the biological role of cadherins expression in GBM dedifferentiation, dissemination or disease aggressiveness are thus far mostly contradictory and, as a consequence, highly inconclusive. In order to further clarify this topic, we herein present the analysis of cadherins expression in a large series of GBM and correlate their expression profiles with clinical, imaging and survival data. In addition, we explored cadherins expression interplay and how it could improve the understanding of GBM exquisite unknown pathogenesis.

Materials and Methods

Patients and materials

A retrospective series of 313 patients who underwent biopsy or surgical resection for newly diagnosed glioblastomas (GBM), between 2010 and 2017, at the Neurosurgery Department, Centro Hospitalar de Santo António (Porto, Portugal), has been retrieved (**Supplementary Figure 1)**. Formalin-fixed and paraffin-embedded (FFPE) tumor tissues were histologically analyzed for all primary tumors, as well as for matched local recurrences in 38 patients. Clinical data, treatment decision, patient and survival follow-up data were extracted by patient's records review for all the cases included in the series. Initial MRI was also evaluated and imaging characteristics documented. This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, as well as under the international Helsinki declaration. Moreover, it has also been approved by the ethics committee at Centro Hospitalar de Santo António (DEFI 104-17 (093-DEFI/091-CES). Study design has been illustrated in **Supplementary Figure 2**.

Neuropathological evaluation

All cases were reviewed by an experienced neuropathologist, blinded to clinical and demographical details. Neuropathological features were analyzed in hematoxylin and eosin staining from the initial tumor samples. Neuropathological phenotypes were evaluated and the presence of distinct areas of differentiation were documented according to the following features: 1) the glial component, which included agglomerates of fibrillary astrocytes, gemistocytes, multinucleated giant cells or cells with oligodendroglial features; 2) the epithelioid component, which has been considered when morphologic epithelial appearance, with large and polygonal shape cells were present, with abundant cytoplasm and defined cell border; 3) a sarcomatous component was also considered, whenever areas of sarcomatous or mesenchymal differentiation were observed, often comprising chondral or osteochondral elements. The predominant component (>50% of the tumor) was documented as well.

Immunohistochemical Staining

Immunohistochemical (IHC) staining was performed with specific monoclonal antibodies for the classical cadherins N-, E-, and P-cadherins in 3 μm sections: E-cadherin (1:100, rabbit, clone 24E19, Cell Signalling, USA), P-cadherin (1:50, mouse, clone C56, BD Transduction, USA) and N-cadherin (1:100, mouse, clone 3B9, ThermoFisher, USA). Briefly, dewaxing and epitope recovery (ER) was performed using Dewax and HIER Buffer M (Epredia, Kalamazoo, USA) and all ER were heat-induced in an Epredia PT Module, according to the standard protocol. The downstream IHC protocol was performed in the Lab VisionTM Autostainer 480S, using the Epredia UltraVision™ Quanto-HRP Detection System. The time for primary antibody incubation was 60 minutes, with the exception for the anti-P-cadherin antibody, where a 90 minutes incubation was performed. All reactions were revealed with 3,3'-diaminobenzidine (DAB) chromogen. Tissues were then counterstained with HIGHDEF® hematoxylin (Enzo, Farmingdale, USA), dehydrated and coverslipped using a permanent mounting solution (Entellan®). Positive and negative controls were included in every set of reactions for each antibody used. The expression for each cadherin was evaluated according to staining intensity [+: weak (1), ++: moderate (2), +++: strong (3)], staining area [<25% (1), 25-50% (2), >50% (3)] and membrane staining [positive (2) or negative (1)]. All the analyses were blinded for clinical and demographical details. An algorithm was then used with the sum of intensity, extension and membrane positivity values, as defined in **Supplementary Table 1.** Whenever the cadherin expression score was > 3, it was considered positive. This scoring system was applied for all cadherins. In tumors comprising various paraffin blocks, a tumor was codified as positive for a given cadherin if positivity was observed at least in one block. In addition to consider isolated

positivity for E-, P- and N-cadherin, we further classified the tumors according to the expression of the 3 cadherins in 8 subgroups: 1) isolated N-cadherin expression; 2) isolated E-cadherin expression; 3) isolated P-cadherin expression; 4) E- and N-cadherin co-expression; 5) P- and N-cadherin co-expression; 6) E- and P-cadherin co-expression; 7) positivity for the 3 cadherins (triple-positive); and 8) negativity for the 3 cadherins (triple-negative).

Data mining from publicly available datasets

RNA expression from a total of 154 glioblastoma multiform patients (TCGA-GBM) were downloaded from genomic data commons along with the available clinical information on 3rd April 2023. Expression data in FPKMs (fragments per kilobase of exon per million mapped fragments) for *CDH1*, *CDH2* and *CDH3* were extracted from the RNA counts file provided by TCGA. Additional clinical information (MGMT status, Methylation class, G-CIMP methylation, IDH1 status, expression subclass and therapy class) were extracted from the original paper (47). All the analyses performed in this report were carried in R version 4.1.2. Expression was classified into high and low categories using the median value of expression as the threshold.

Statistics

The chi-square test was used to assess group differences, as appropriate. Overall survival from GBM diagnosis to patient death or last follow-up was estimated, using the Kaplan-Meier limit method. The log-rank test was used to assess group differences. A 2-tailed significance level (p-value) of 0.05 was applied. Graph Pad Prism version 9.0c software (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis and graphical data presentation.

Results

Clinical and neuropathological characteristics of glioblastoma series

Patient characteristics, including clinical baseline parameters and treatment decisions, are detailed in **Supplementary Table 2.** The series of 313 GBM patients include a similar number of male (n=178; 56.9%) and female (n=135; 43.1%) individuals, with a mean age at diagnosis of 61,743 ± 10,483 years old. Regarding presentation at diagnosis, the majority of patients had focal deficits (n=116; 37.1%), followed by headaches (n=74; 23.6%), behaviour changes (n=65; 20.8%), and seizures (n=54; 17.3%). The mean for the initial functional status, measured by the Karnofsky Performance Scale (KPS), was 79.33 ± 10.585. Concerning tumor MRI characteristics, we could describe that the mean major axis of the tumors was 7.27 ± 2.09 cm. The majority were unilobar (n=242; 77.3%), mostly located in the frontal (36.1%) and temporal lobes (30.4%), without ependymal (n=224; 71.6%) or corpus callosum extension (n=221; 70.6%). Less than a third of the tumors (n=76, 24.3%) were multifocal and 49 (15.7%) presented cysts. The majority of patients underwent surgical resection (n=177, 56.5%), followed by chemotherapy (temozolomide) and radiotherapy, as proposed by Stupp *et al.* (64.9%)(48). We documented tumor recurrence or regrowth in 297 patients. Of these, 38 patients underwent surgical resection and 159 patients underwent 2nd line treatments.

Neuropathologic features analyzed are summarized in **Table 1**. Necrosis and vascular changes were ubiquitous in primary tumors. We identified the presence of inflammation in about a third of the cases (n=80; 25.6%). A glial component was present in up to 99.7% of the tumors (n=312), in particular gemistocytes were identified in 15.7% (n=49) and an oligodendroglial component in 11.7% (n=36). A mesenchymal component was identified in 20.1% of the tumors (n=63), more commonly than the presence of an epithelial component, which was only found in 13.1% (n=41). Based on the outlined criteria, tumors were divided in predominantly glial (n=291; 93%), predominantly mesenchymal (n=15; 4.8%), or predominantly epithelial (n=7; 2.2%).

Table 1. Individual Cadherins expression in GBM and association with neuropathological features.

	GBM series	**N-cadherin positive**		**E-cadherin positive**		**P-cadherin positive**	
	n (%)	**n (%)**	***p-value***	**n (%)**	***p-value***	**n (%)**	***p-value***
	313 (100%)	255 (81.5%)		97 (31%)		65 (20.8%)	
Papillary structures	17 (5.4%)	15 (5.9%)	*0.46*	17 (17.5%)	***<0.001***	6 (9.2%)	*0.126*
Whorls	8 (2.6%)	5 (2%)	*0.162*	4 (4.1%)	*0.239*	6 (9.2%)	***<0.001***
Glomeruloid vessels	47 (15%)	39 (15.3%)	*0.773*	20 (20.6%)	*0.063*	9 (13.8%)	*0.767*
Myxoid stroma	18 (5.7%)	16 (6.3%)	*0.404*	9 (9.3%)	*0.072*	4 (6.2%)	*0.875*
Inflammation	80 (25.6%)	69 (27%)	*0.502*	39 (40.2%)	***0.001***	33 (50.8%)	***<0.001***
Calcifications	10 (3.2%)	10 (3.9%)	*0.125*	3 (3.1%)	*0.945*	0	*0.1*
Oligodendroglial component	36 (11.7%)	32 (12.5%)	*0.643*	9 (9.3%)	*0.299*	0	***0.014***
Gemistocytes	49 (15.7%)	49 (19.5%)	***<0.001***	13 (13.4%)	*0.424*	5 (7.8%)	*0.058*
Glial component	312 (99.7%)	254 (99.6%)	*0.633*	96 (99%)	*0.135*	65 (100%)	*0.608*
Mesenchymal component	63 (20.1%)	44 (17.3%)	*0.75*	25 (25.8%)	***0.005***	43 (66.2%)	***<0.001***
Epithelial component	41 (13.1%)	36 (14.1%)	*0.263*	39 (40.2%)	<***0.001***	15 (23%)	*0.007*
Predominant component							
Glial	291 (93%)	242 (94.9%)	*0.981*	86 (88.7%)	***<0.001***	53 (81.5%)	***0.001***
Mesenchymal	15 (4.8%)	8 (3.1%)	*0.561*	4 (4.1%)	*0.376*	9 (13.8%)	***<0.001***
Epithelial	7 (2.2%)	5 (2%)	*0.489*	7 (7.2%)	***<0.001***	3 (4.6%)	*0.145*
Tumor recurrence	38	37 (97.4%)		8 (20.5%)		25 (65.8%)	

Cadherins expression associate with clinical, imaging and neuropathological GBM features

We have studied the expression of the classical cadherins, E-, N- and P-cadherin, in the series of 313 GBMs **(Figure 1A)**. In cases where preserved normal brain parenchyma was present, we observed N-cadherin expression in neurons and ganglion cells, as expected; however, no staining was found in the brain parenchyma for neither E- or P-cadherin **(Figure 1A)**.

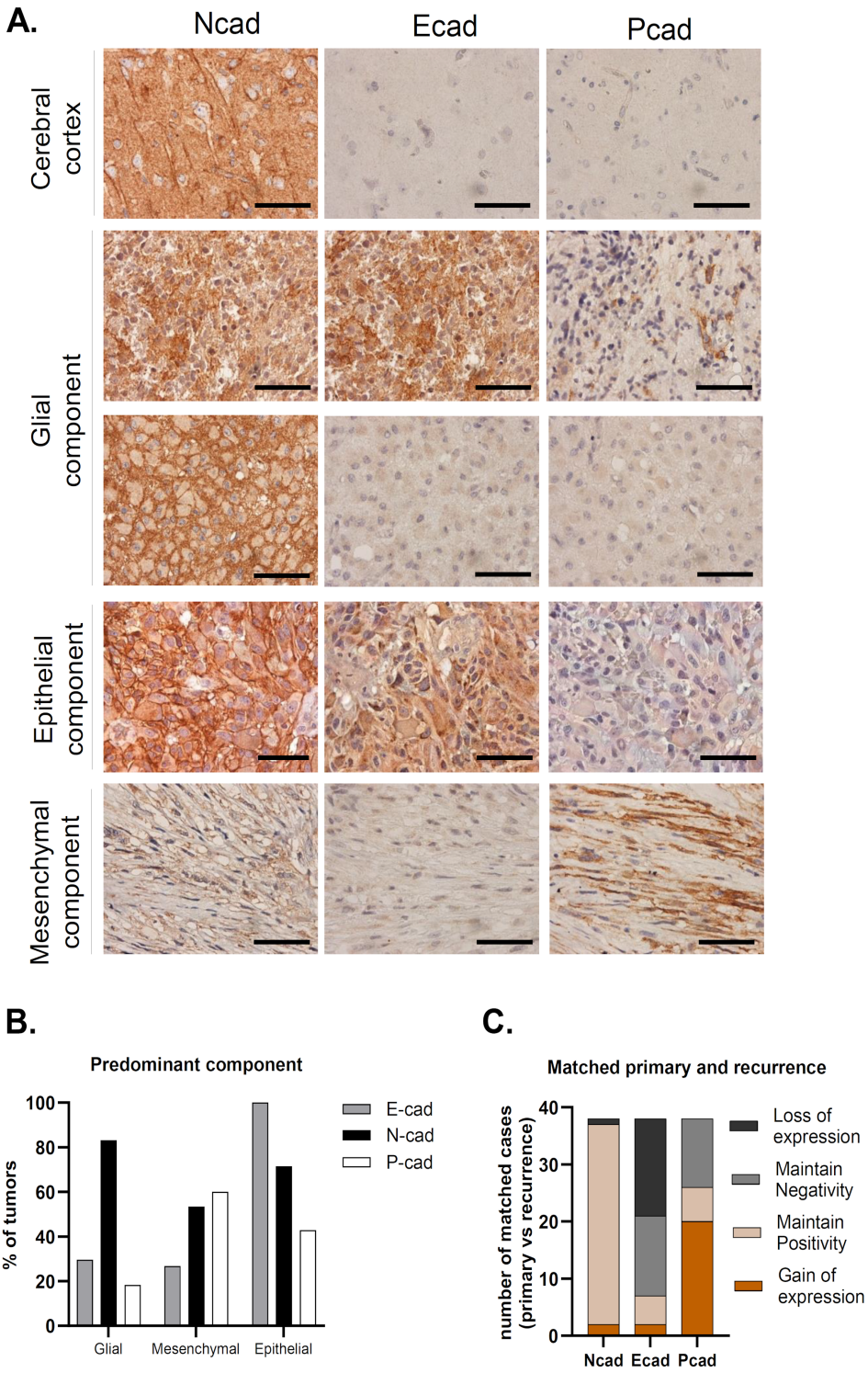

Figure 1. N-, E- and P-Cadherin expression in Glioblastoma. (A) Representative cases of predominant glial, epithelial and mesenchymal component tumors. In tumor with a predominant glial component, N-cadherin positivity was observed in the majority of the cases and frequently strong, with membrane staining. In some cases, E-cadherin positivity was also observed. Tumors with a predominant epithelial component stained for E-cadherin in all cases, which was frequently accompanied by N-cadherin staining as observed in this case. Finally, tumors with a predominant mesenchymal component most commonly stained for both N-cadherin and P-cadherin (Scale bar corresponds to 50 μm). (B) N-cadherin expression is high in all tumor subtypes. In tumors with a predominant mesenchymal component, P-cadherin and N-cadherin expression is observed in the vast majority of cases. Strikingly, all cases with a predominant epithelial component are positive for E-cadherin. (C) Upon tumor recurrence, 97.4% of the tumors are positive for N-cadherin (maintain positive + gain of positivity). Gain of P-cadherin expression was the most common event which was observed in over half of the cases (51.3%). This leads to an important increase in P-cadherin positivity in recurrence when compared to the primary tumor (20.8% vs 65.8%). On the contrary, E-cadherin positivity is lower in recurrence (31% vs 20.8%), due to E-cadherin loss of staining in matched primary-recurrent cases (43.6%).

N-cadherin was the most commonly expressed cadherin in up to 81.5% of the tumor samples (n=255) (**Table 1)**. The majority of these samples had strong N-cadherin staining, since compound scores were higher than 5 in 49.9% of the cases (n=156). Moreover, membrane staining was observed in 64.5% (n=202). In the available matched recurrence cases, virtually all tumors were N-cadherin positive (n=37; 97.4%), where a strong staining was observed in 77% of the cases (n=30) **(Table 1, Figure 1C)**. Interestingly, N-cadherin was ubiquitously expressed among tumors with different neuropathological components: glial (254 in 312 cases, 81%), mesenchymal (44 in 63 cases, 70%) and epithelial (36 in 41 cases, 88%) **(Table 1, Figure 1B)**. Importantly, N-cadherin expression did not associate significantly with any particular patient or tumor characteristic.

Positivity for E-cadherin was only found in 31% (n=97) of the cases (**Table 1)**. In particular, E-cadherin staining was more frequent in the cytoplasm of larger cells, with an abundant cytoplasm and defined cell borders, with occasional membrane staining (10.3%) **(Figure 1A)**. Only 9.4% of the cases had compound scores superior to 5 (n=31). Upon tumor recurrence, contrary to what was observed for N-cadherin, E-cadherin staining loss was observed in a large number of re-operated patients with initial positivity for E-cadherin (43.6%). E-cadherin positivity dropped to 20.5% (n=8) in the recurrent cases **(Table 1, Figure 1C)**. Interestingly, E-cadherin positive tumors were more frequently observed in younger patients (46.7% positivity in patients <43 years old, *p=0.079*), characteristically located in the temporal lobe (48%, *p=0.007*), with the presence of cysts (*p=0.028*) and a tendency for cortical involvement (*p=0.094*). The neuropathological characteristics that significantly correlated with immunostaining for E-cadherin included the presence of papillary structures (*p<0.001*) and the presence of inflammatory infiltrates (*p=0.001*). Strikingly, E-cadherin was positive in 95.1% (39 in 41 cases) of the cases with an epithelial component **(Table 1)**. In cases with a predominant epithelial component, E-cadherin was consistently expressed in all cases (7 in 7 cases) (*p<0.001*). On the other hand, E-cadherin was positive in 26.7% (4 in 15 cases) of the cases with predominant mesenchymal differentiation and in 29.5% (86 in 291 cases) of the cases with predominant glial differentiation **(Table 1, Figure 1B)**.

Immunostaining for P-cadherin was only found in 20.8% of primary GBM (n=65) **(Table 1).** As for E-cadherin, P-cadherin was also found in the cytoplasm of large cells, with low membrane staining (6.1% of the cases) **(Figure 1A)**. In addition, it commonly appeared in tumors containing fusiform cells **(Figure 1A)**. Strikingly, P-cadherin positivity rate in recurrence was significantly higher compared to the primary tumors (65.8% vs 20.8%) **(Table 1, Figure 1C)**. Comparing matched primary and recurrent tumors, P-cadherin gain was the most frequent event, present in 51.3% of the tumor matched pairs. The presence of a mesenchymal component also significantly correlated with positive staining for P-cadherin (*p<0.001*), which was positive in up to 66% of these cases. It was also positive in 23% of the cases with an epithelial component **(Table 1, Figure 1B)**. Indeed, positivity for P-cadherin was found in 60% of the tumors where the mesenchymal component was predominant (9 in 15 cases), in 42.9% where there was a predominance of the epithelial component (3 in 7 cases), and in 18.2% of predominant glial tumors (53 in 291 cases) (*p<0.001*). In addition, we identified the presence of lymphocytic infiltrates in about 50% of P-cadherin positive cases (*p<0.001*).

Cadherins co-expression profiles improve the identification of GBM subgroups

We further classified tumors into 8 subgroups according to the co-expression of the three studied cadherins. The expression of more than one cadherin was identified in 124 tumors (39.6%), while only 22 tumors expressed all the three cadherins (triple positive tumors, 7%). Moreover, the immunostaining was negative for N-, E- and P-cadherin in 40 tumors (12.8%, triple negative tumors). Most commonly, tumors expressed only N-cadherin (n=137, 43.8%) or co-expressed E- and N-cadherin (n=67, 21.4%), as shown in **Table 2 and Figure 2A**. Interestingly, upon tumor recurrence, the most common profiles were the co-expression of P- and N-cadherin (46.2%) or the isolated expression of N-cadherin (30.8%) **(Figure 2A)**. Cadherins expression profiles changed in 69.2% of the cases. Curiously, all E-cadherin positive cases, upon tumor recurrence, showed concomitant expression of P- and N-cadherin **(Figure 2A)**.

Table 2. Cadherins co-expression profiles in glioblastomas and association with clinical, imaging and neuropathological characteristics.

	GBM series	**Ncad**		**Ecad**		**Pcad**	
	n (%)	**n (%)**	***p* value**	**n (%)**	***p* value**	**n (%)**	***p* value**
	313 (100%)	137 (43.8%)		4 (1.3%)		8 (2.6%)	
Presentation							
Focal deficits	116 (37.1%)	52 (38%)	*0.773*			6 (75%)	***0.024***
Headache	74 (23.6%)	24 (17.5%)	***0.024***	3 (75%)	***0.015***		
Behaviour	65 (20.8%)	35 (25.5%)	*0.066*	1 (25%)	*0.834*	2 (25%)	*0.766*
Seizures	54 (17.3%)	23 (16.8%)	*0.849*				
Finding	4 (1.3%)	3 (2.2%)	*0.206*				
Age (median)	61.743 ± 1.483	62.1± 11.0	*0.848*	49 ± 16.39	***0.015***	66.25 ± 6.409	*0.299*
Location							
Frontal	113 (36.1%)	59 (43.1%)	***0.024***	1 (25%)	*0.643*	3 (37.5%)	*0.934*
Temporal	95 (30.4%)	29 (21.2%)	***0.002***	1 (25%)	*0.815*	3 (37.5%)	*0.657*
Parietal	70 (22.4%)	33 (24.1%)	*0.52*	1 (25%)	*0.899*	1 (12.5%)	*0.499*
Deep	15 (4.8%)	6 (4.4%)	*0.764*	1 (25%)	*0.057*	1 (12.5%)	*0.303*
Other	20 (6.4%)	10 (7.3%)	*0.563*				
Cysts	49 (15.7%)	33 (24.1%)	*0.65*	2 (50%)	*0.057*	0	*0.218*
Multifocal	76 (24.3%)	35 (25.5%)	*0.645*	1 (25%)	*0.973*	5 (62.5%)	***0.011***
Corpus callosum ext.	92 (29.4%)	40 (29.2%)	*0.946*	1 (25%)	*0.846*	4 (50%)	*0.195*
Ependymal extension	89 (28.4%)	39 (28.5%)	*0.991*	2 (50%)	*0.336*	5 (62.5%)	***0.03***
Predominant component							
Glial	291 (93%)	135 (97.8%)	***0.003***	2 (50%)	***<0.001***	7 (87.5%)	*0.541*
Mesenchymal	15 (4.8%)	3 (2.2%)	*0.057*			1 (12.5%)	*0.303*
Epithelial	7 (2.2%)			2 (50%)	***<0.001***		
Treatment							
No	96 (30.7%)	46 (33.6%)	0.327			3 (37.5%)	0.673
RT	19 (6.1%)	7 (5.1%)	0.531	1 (25%)	0.111		
QT+RT	8 (2.5%)	3 (2.2%)	0.718				
Stupp	189 (60.4%)	81 (59.1%)	0.615	3 (75%)	0.557	5 (62.5%)	0.916

Table 2. (cont)

	Ecad/Ncad		Pcad/Ncad		Ecad/Pcad		Triple positive		Triple negative	
	n (%)	***p value***	**n (%)**	***p value***	**n (%)**	***p value***	**n (%)**	***p value***	**n (%)**	***p value***
	67 (21.4%)		29 (9.3%)		6 (1.9%)		22 (7%)		40 (12.8%)	
Presentation										
Focal deficits	17 (25.4%)	***0.025***	12 (41.4%)	*0.615*	5 (83.3%)	***0.018***	8 (36.4%)	*0.944*	16 (40%)	*0.681*
Headache	19 (28.4%)	*0.307*	10 (34.5%)	*0.15*			6 (27.3%)	*0.679*	12 (30%)	*0.312*
Behaviour	13 (19.4%)	*0.757*	4 (13.8%)	*0.333*	1 (16.7%)	*0.803*	4 (18.2%)	*0.757*	5 (12.5%)	*0.169*
Seizures	18 (26.9%)	***0.019***	3 (10.3%)	*0.303*			3 (13.6%)	*0.643*	7 (17.5%)	*0.965*
Finding							1 (4.5%)	*0.158*		
Age (median)	60 ± 11.6	*0.264*	61.10 ± 8.491	*0.55*	68.83 ± 5.037	*0.139*	62.2 ± 10	*0.97*	65.4 ± 11.6	*0.055*
Location										
Frontal	23 (34.3%)	*0.734*	9 (31%)	*0.552*	1 (16.7%)	*0.367*	5 (22.7%)	*0.177*	12 (30%)	*0.391*
Temporal	28 (41.8%)	***0.022***	11 (37.9%)	*0.353*	1 (16.7%)	*0.463*	10 (45.5%)	*0.111*	12 (30%)	*0.959*
Parietal	11 (16.4%)	*0.189*	5 (17.2%)	*0.489*	4 (66.7%)	***0.008***	5 (22.7%)	*0.966*	10 (25%)	*0.67*
Deep	2 (3%)	*0.436*	1 (3.4%)	*0.723*			1 (4.5%)	*0.955*	3 (7.5%)	*0.392*
Other	3 (4.5%)	*0.472*	3 (10.3%)	*0.362*			1 (4.5%)	*0.715*	3 (7.5%)	*0.759*
Cysts	22 (32.8%)	***<0.001***	1 (3.4%)	*0.057*	2 (33.3%)	*0.229*	0	***0.036***	2 (5%)	***0.047***
Multifocal	10 (14.9%)	***0.044***	12 (41.4%)	***0.024***	2 (33.3%)	*0.602*	6 (27.3%)	*0.734*	5 (12.5%)	*0.063*
Corpus callosum ext.	17 (25.4%)	*0.415*	13 (44.8%)	*0.055*	2 (33.3%)	*0.831*	7 (31.8%)	*0.796*	8 (20%)	*0.163*
Ependymal extension	14 (20.9%)	*0.123*	9 (31%)	*0.745*	4 (66.6%)	***0.036***	7 (31.8%)	*0.715*	9 (22.5%)	*0.373*
Predominant component										
Glial	63 (94%)	*0.703*	22 (75.9%)	***<0.001***	6 (100%)	*0.498*	17 (77.3%)	***0.003***	40 (100%)	*0.063*
Mesenchymal			7 (24.1%)	***<0.001***			4 (18.2%)	***0.002***		
Epithelial	4 (6%)	***0.020***					1 (4.5%)	*0.449*		
Treatment										
No	13 (19.4%)	0.086	11 (37.9%)	0.375	1 (16.7%)	0.454	7 (31.8%)	0.904	15 (37.5%)	0.317
RT	3 (4.5%)	0.540	1 (3.4%)	0.536	1 (16.7%)	0.274			6 (15%)	***0.011***
QT+RT	2 (3%)	0.803	1 (3.4%)	0.750					2 (5%)	0.296
Stupp	49 (73.1%)	0.079	16 (55.2%)	0.524	4 (66.7%)	0.764	15 (68.2%)	0.458	17 (42.5%)	0.096

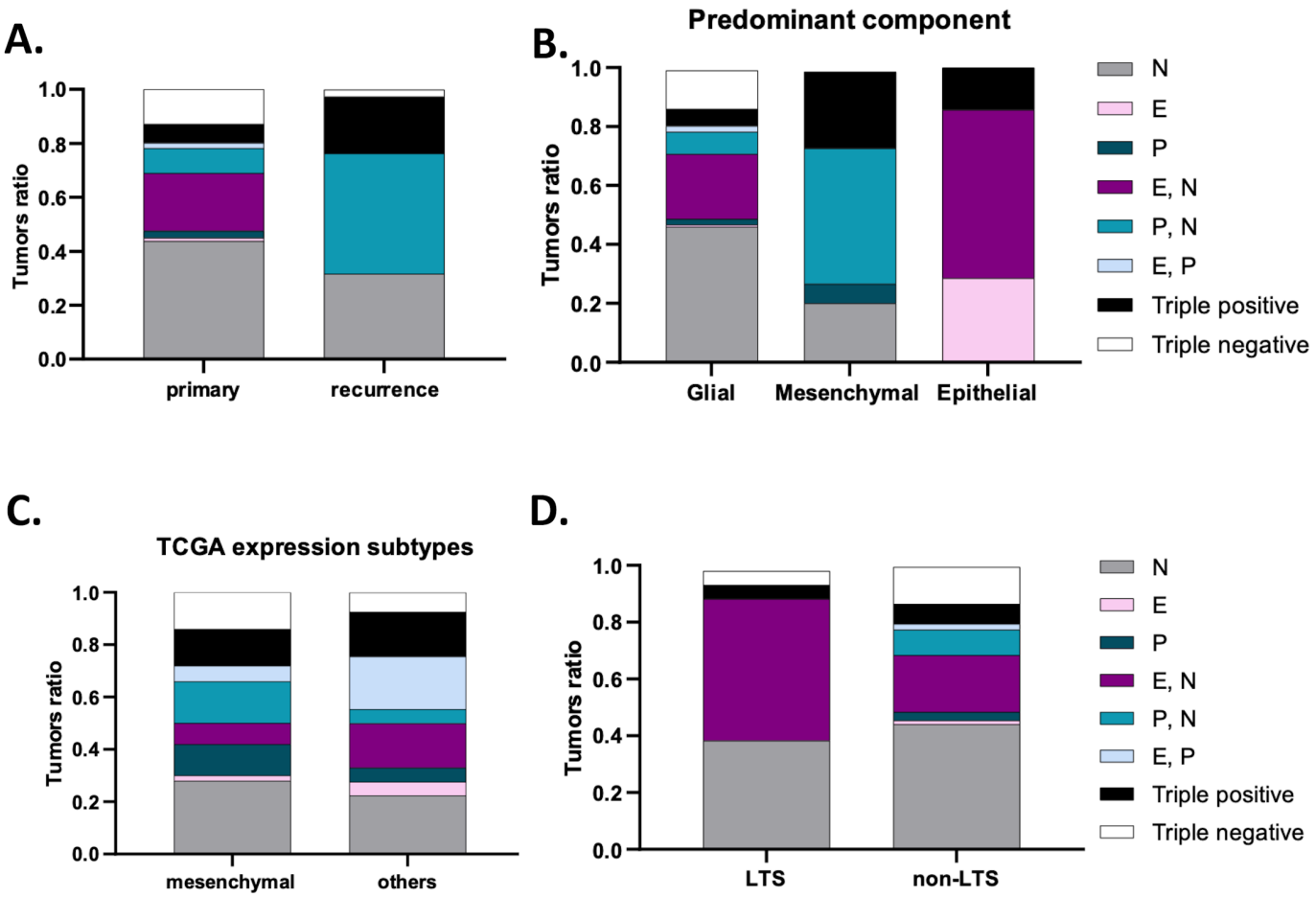

Figure 2. Cadherin expression subgroups in glioblastoma. (A) Compared to primary tumors, the most frequent subgroups in recurrent tumor are P- and N-cadherin co-expression (46.2%) and N-cadherin expression (30.8%). (B) Tumors with a predominant epithelial component expressed E-cadherin, E- N- cadherin or triple positive subgroups. For mesenchymal tumor, the subgroups expressed were P- N- cadherin, triple positive, P-cadherin and N- cadherin. (C) Analysis of data from the TCGA, confirmed a main expression of P-N cadherin, N-cadherin, triple positive and N-cadherin subgroups in the mesenchymal transcriptional subtype. P-N- co-expression significantly correlated with the mesenchymal subtype (p=0.033). (D) Tumors from long-term survival patients exhibited more frequently E-N-cadherin co-expression, N-cadherin, triple positive or triple negative subgroups (*p=0.013*).

In tumors with more than one FFPE block, we evaluated the heterogeneity of each cadherin subgroup. Over 50% of the tumors showed intratumoral cadherin expression heterogeneity, including 4 distinct subgroups in 1.7% of the tumors. Curiously, N-cadherin expression was heterogeneous in 40% of the tumors and E-cadherin in 28% of the tumors, while P-cadherin was heterogeneous in only 16% of the tumors.

When considering the combined expression of the three cadherins, we further evaluated putative significant correlations with clinical, imaging and neuropathological characteristics. Clinical presentation associated to cadherin co-expression profiles **(Table 2)**. In particular, patients with tumors that expressed epithelial cadherins more frequently presented focal deficits (P-cadherin, *p=0.024* and co-expression of E and P-cadherin, *p=0.018*); while headache was the most common presentation in patients with tumors that showed isolated E-cadherin expression (*p=0.015*). These expression profiles also correlated with age, specifically the isolated expression of E-cadherin was more common in younger patients (*p=0.015*). Tumor location was more common in the frontal lobe for isolated N-cadherin expression tumors (*p=0.024*) and the temporal lobe for the co-expression of E- and N-cadherin (*p=0.022*). Other imaging features correlated with cadherin expression subgroups included: cyst formation (co-expression E and N-cadherin, *p<0.001*; and a tendency for E-cadherin, *p=0.057*), multifocality (P-cadherin expression, p=*0.011*; and co-expression P- and N-cadherin, *p=0.024*), ependymal extension (P-cadherin

expression, *p=0.030*; and E- and P-cadherin co-expression, *p=0.036*), and corpus callosum extension (tendency for P- and N- cadherin co-expression, *p=0.055*).

When evaluating for distinct neuropathological phenotypes, we observed that tumors with a predominant sarcomatous/mesenchymal differentiation predominantly exhibited N- and P-cadherin co-expression (44.4%), or were positive for the 3 cadherins (33.3%), or expressed only P-cadherin (11.1%) or only N-cadherin (11.1%) (*p<0.001*) **(Figure 2B)**. Predominant epithelial differentiation was observed in tumors showing only E-cadherin expression (28.6%), E- and N-cadherin co-expression (28.6%) and the expression of the 3 cadherins (triple positive, 42.9%) (*p<0.001*) **(Figure 2B)**. Tumors with predominant glial expression had most frequently only N-cadherin expression (46.1%), E- and N-cadherin co-expression (21.5%) or were negative for the 3 cadherins (13.5%) **(Figure 2B)**.

Based on phenotypic differentiation results, we further explored cadherin expression differences according to transcription subtypes, particularly the mesenchymal subtype, using the online available TCGA data. Indeed, this data showed that tumors with mesenchymal transcription subtype were enriched for isolated N-cadherin expression (28%), P- and N-cadherin co-expression (16%), triple positivity (14%), triple negativity (14%) and isolated P-cadherin expression (12%) subgroups. When compared to tumors with other subtypes, P- and N-cadherin co-expression specifically associated with the presence of a mesenchymal subtype (*p=0.019*) **(Figure 2C)**.

Cadherins expression profiles define GBM patients' prognosis

As expected, patients' age (HR=1.405; *p=0.004*), functional status (KPS 50; HR=1.772; *p=0.006*), surgical decision (biopsy; HR=2.329; *p<0.001*) and complimentary treatment (no treatment; HR=4.73; *p<0.001*) were validated as prognostic factors in this particular GBM series **(Table 3)**. Thus, survival analysis was used to correlate cadherins expression with GBM patients' prognosis.

In particular, N-cadherin expression identified tumors with improved progression-free survival (median 6 months, log rank test, *p=0.033*) and overall survival (median 12 months, log rank test, *p=0.001*) (**Figure 3A)**, while a significant decrease in progression-free survival and overall survival was observed in tumors positive for P-cadherin expression (log rank test, median 3 months and 7 months, *p=0.028* and *p=0.024*, respectively) (**Figure 3C**). E-cadherin staining identified tumors with a longer time to recurrence (7 months, log rank test, *p=0.008*) and a better overall survival (14 months, log rank test, *p=0.039*) (**Figure 3B**).

Interestingly, the combined expression of cadherins also stratified patients' prognosis, both progression-free and overall survival **(Figure 3D and Table 3)**. For instance, Kaplan-Meier survival curves showed a survival benefit for E- and N-cadherin co-expression for both progression free (log rank test, *p=0.005*, median 7 months) and overall survival (log rank test, 14 months, *p=0.005*) **(Figure 3D)**. On the contrary, P- and N-cadherin or E- and P-cadherin co-expression showed the shorter times to recurrence (median 2 and 3 months, log rank test, *p=0.046* and *p=0.093*, respectively), while E- and P-cadherin co-expression and single P-cadherin expression showed a tendency for worse overall survival (log rank test, 9 months, *p=0.078* and 9 months, *p=0.055,* respectively) **(Figure 3D).**

Univariate analysis identified three subgroups with worse progression free survival: E- and P-cadherin co-expression (HR=2.203, *p=0.035*), P- and N-cadherin co-expression (HR=1.91, *p=0.008*) and triple negative tumors (HR=1.712, *p=0.021*); and four subgroups with worse overall survival: E- and P-cadherin co-expression (HR=2.728, *p=0.020*), P-cadherin (HR=2.611, *p=0.012*), triple negative tumors (HR=1.794, *p=0.005*) and P- and N- co-expression (HR=1.619, *p=0.036*) **(Table 3)**.

Multivariate analysis identified E- and P-cadherin co-expression (HR=2.96, *p=0.020*) as a significant independent factor for worse progression-free survival **(Table 4)**. E-cadherin (HR=4.24, *p=0.017*), E- and P-cadherin co-expression (HR=3.35, *p=0.021*), P-cadherin (HR=2.33, *p=0.036*), and triple positivity (EPN) (HR=2.11, *p=0,011*), were also found as significant independent factors for worse overall survival **(Table 4)**. In concordance with these results, of the 21 long-term survivors (LTS) (6.7%) with in our series, 42.9% were N- and E-cadherin co-expressing tumors, compared to the 19.9% expression in non-LTS (*p=0.013*); 38.1% of the GBM showed N-cadherin expression, 9.5% were triple positive and other 9.5% were triple

negative tumors. Strikingly, the tumors from LTS patients did not show isolated expression of epithelial cadherins (E- and P-cadherins) (**Figure 2D**).

Table 3. Univariate analysis of Cadherins expression profile in glioblastomas and association with patient's prognosis.

		Progression Free Survival				Overall Survival			
			95.0% CI				95.0% CI		
		HR	Inferior	Superior	p-value	HR	Inferior	Superior	p-value
Surgery	**Surgery**	1				1			
	Biopsy	2.120	*1.626*	*2.763*	**<0.001**	2.329	*1.842*	*2.945*	**<0.001**
Treatment	**Yes**	1				1			
	No	13.244	*8.492*	*20.665*	**<0.001**	4.730	*3.547*	*6.308*	**<0.001**
KPS	**100**	1				1			
	50	0.505	*0.056*	*4.550*	0.543	1.772	*1.183*	*2.654*	**0.006**
	60	0.580	*0.078*	*4.318*	0.595	1.411	*0.866*	*2.298*	0.167
	70	0.587	*0.080*	*4.295*	0.600	2.575	*1.223*	*5.421*	**0.013**
	80	0.430	*0.059*	*3.105*	0.403	2.596	*1.035*	*6.508*	**0.042**
	90	0.391	*0.054*	*2.826*	*0.352*	1.599	*1.022*	*2.503*	**0.040**
Age	**< 65 yo**	1				1			
	> 65 yo	1.258	*0.974*	*1.625*	0.079	1.400	*1.113*	*1.762*	**0.004**
Ncad	**Negative**	1				1			
	Positive	0.725	*0.522*	*1.007*	0.055	0.627	*0.468*	*0.840*	**0.002**
Ecad	**Negative**	1				1			
	Positive	0.716	*0.546*	*0.938*	**0.016**	0.779	*0.608*	*0.998*	**0.048**
Pcad	**Negative**	1				1			
	Positive	1.367	*1.003*	*1.863*	**0.048**	1.364	*1.029*	*1.806*	**0.031**
Cadherins co-expression	**Ecad/Ncad**	1				1			
	Ncad	1.367	*0.984*	*1.900*	0.063	1.316	*0.969*	*1.787*	0.079
	Ecad	1.507	*0.471*	*4.826*	0.490	2.550	*0.923*	*7.041*	0.071
	Pcad	1.864	*0.884*	*3.933*	0.102	2.611	*1.239*	*5.502*	**0.012**
	Pcad/Ncad	1.910	*1.186*	*3.076*	**0.008**	1.619	*1.033*	*2.536*	**0.036**
	Ecad/Pcad	2.203	*1.068*	*5.868*	**0.035**	2.728	*1.169*	*6.366*	**0.020**
	Triple positive	1.194	*0.683*	*2.087*	0.533	1.427	*0.875*	*2.328*	0.154
	Triple negative	1.712	*1.084*	*2.704*	**0.021**	1.794	*1.196*	*2.690*	**0.005**

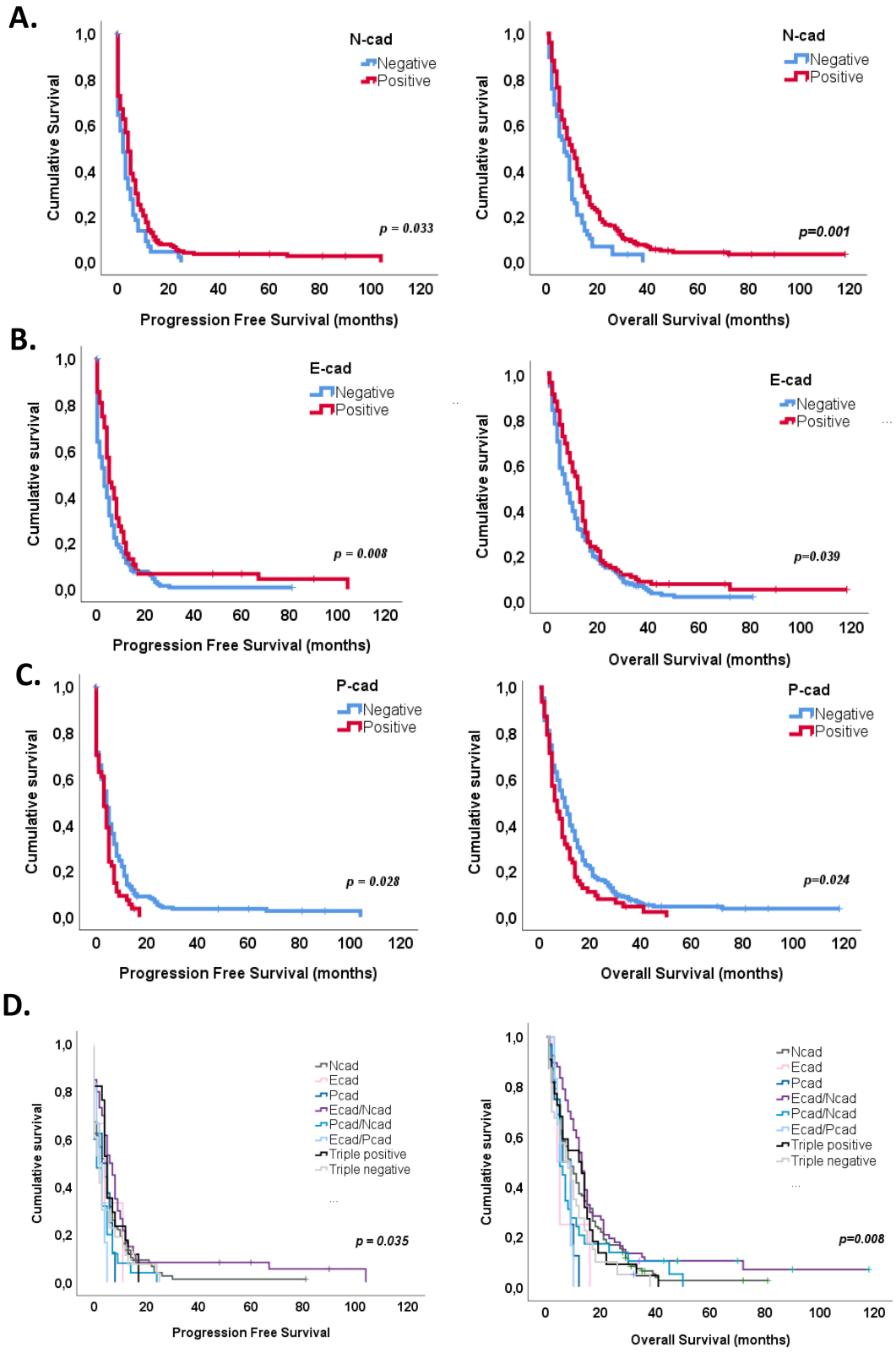

Figure 3. Survival analysis according to cadherins expression. (A) N-cadherin expression in GBM showed a significant improve in both progression-free (*p=0.033*) and overall survival (*p=0.001*). (B) A significant survival benefit was also observed in tumors with E-cadherin expression (*p=0.008* for progression-free survival and *p=0.039* for overall survival. (C) On the contrary, P-cadherin expression significantly marked tumors with a shorter time for recurrence (*p=0.028*) and survival (*p=0.024*). (C) Kaplan-Meier curves identified a survival benefit for E-N- cadherin co-expression subgroup (*p=0.005* for both PFS and OS). P-N-cadherin co-expression (*p=0.046*) subgroup significantly associated to a worse progression-free survival, while E- P- cadherin co-expression (*p=0.078*) and P-cadherin (*p=0.055*) subgroups showed a tendency for worse overall survival.

Multivariate analysis identified E- and P-cadherin co-expression (HR=2.96, *p=0.020*) as a significant independent factor for worse progression-free survival **(Table 4)**. E-cadherin (HR=4.24, *p=0.017*), E- and P-cadherin co-expression (HR=3.35, *p=0.021*), P-cadherin (HR=2.33, *p=0.036*), and triple positivity (EPN) (HR=2.11, *p=0,011*), were also found as significant independent factors for worse overall survival **(Table 4)**. In concordance with these results, of the 21 long-term survivors (LTS) (6.7%) with in our series, 42.9% were N- and E-cadherin co-expressing tumors, compared to the 19.9% expression in non-LTS (*p=0.013*); 38.1% of the GBM showed N-cadherin expression, 9.5% were triple positive and other 9.5% were triple negative tumors. Strikingly, the tumors from LTS patients did not show isolated expression of epithelial cadherins (E- and P-cadherins) (**Figure 2D**).

Table 4. Multivariate analysis of Cadherins expression profile in glioblastomas and association with patient's prognosis.

		Progression Free Survival				**Overall Survival**			
		HR	**95.0% CI**		**p-value**	**HR**	**95.0% CI**		**p-value**
			Inferior	**Superior**			**Inferior**	**Superior**	
Mesenchymal Component	**yes**	1.000				1.000			
	no	1.060	*0.670*	*1.660*	0.811	0.810	*0.530*	*1.250*	0.345
Epithelial Component	**yes**	1.000				1.000			
	no	0.930	*0.580*	*1.470*	0.743	0.720	*0.450*	*1.140*	0.160
Cadherins co-expression	**Ecad/N-cad**	1.000				1.000			
	Ncad	0.990	*0.690*	*1.440*	0.979	1.300	*0.900*	*1.870*	0.162
	Ecad	2.200	*0.650*	*7.430*	0.205	4.240	*1.290*	*13.930*	**0.017**
	Pcad	1.330	*0.600*	*2.980*	0.482	2.330	*1.050*	*5.130*	**0.036**
	Pcad/Ncad	1.340	*0.770*	*2.360*	0.303	1.440	*0.850*	*2.460*	0.178
	Ecad/Pcad	2.960	*1.180*	*7.400*	***0.020***	3.350	*1.330*	*8.440*	**0.010**
	Triple positive	1.190	*0.640*	*2.210*	0.577	2.110	*1.190*	*3.760*	**0.011**
	Triple negative	1.090	*0.660*	*1.820*	0.728	1.490	*0.910*	*2.450*	0.113
Surgery	**Surgery**	1.000				1.000			
	Biopsy	1.760	*1.290*	*2.390*	**<0.001**	1.550	*1.170*	*2.060*	**0.003**
Treatment	**yes**	1.000				1.000			
	no	15.000	*9.020*	*24.930*	**<0.001**	4.570	*3.300*	*6.330*	**<0.0001**
KPS	**100**	1.000				1.000			
	50	0.150	*0.020*	*1.390*	0.094	0.700	*0.080*	*6.170*	0.752
	60	0.250	*0.030*	*1.900*	0.179	2.400	*0.320*	*18.040*	0.396
	70	0.240	*0.030*	*1.790*	0.162	2.190	*0.300*	*16.220*	0.442
	80	0.210	*0.030*	*1.550*	0.125	1.520	*0.210*	*11.150*	0.681
	90	0.200	*0.030*	*1.510*	0.118	1.310	*0.180*	*9.660*	0.790
Age	**< 65 yo**	1.000				1.000			
	> 65 yo	1.210	*0.920*	*1.600*	0.176	1.270	*0.980*	*1.660*	0.072

Discussion

E-cadherin, P-cadherin and N-cadherin expression function and impact in prognosis is well established in various systemic cancers. It is nevertheless important to remember that this consensus is accepted for epithelial tumors and, although E- to N-cadherin switch is frequently used as a marker of EMT in cancers of other origins, the factual involvement of these proteins in processes akin to the EMT in non-epithelial tumors remains to be adequately studied and clarified. Actually, the EMT process became recently accepted as a continuum between these two definable states, often achieving hybrid phenotypes, with increased cell plasticity. In this context, cadherin expression regulation will most likely follow the cell-cell adhesion requirements of the different EMT states.

Given the knowledge gap in cadherin expression in glial tumors, our main goal was to characterize the expression of the most studied classical cadherins (E-, N- and P-cadherins) in a large and representative series of GBM. To do so, and taking into account GBM intratumoral heterogeneity, we marked the expression of these proteins in all available paraffin blocks of 313 patients, in a total of 727 samples. Our main findings were the following: 1) N-cadherin is the most frequent cadherin expressed in GBM, present in over 80% of the cases, followed by E-cadherin in about a third of the tumors and P-cadherin in 20%; 2) expression of epithelial cadherins (E- and P-cadherin) determines a distinct imaging and neuropathological profile in GBM; and 3) expression of epithelial cadherins have a significant detrimental impact in GBM patient's prognosis. In addition to showing distinct associations with clinical and neuropathological characteristics, it is notable the distinct behavior of E- and P-cadherins expression upon tumor recurrence. There was a clear P-cadherin dominance and a loss of E-cadherin expression in recurrent lesions compared with primary tumors. Survival analysis further supported P-cadherin as a marker of tumor aggressiveness, as P-cadherin expressing tumors were significantly correlated with a worse patient prognosis. On the contrary, E-cadherin expression was observed in patients with a significant increase in progression-free and overall survival.

These results, although corroborated by robust morphological correlations, only considered the expression of each isolated cadherin and may thus represent a simplified version of an intricately regulated cellular mechanism between them. In fact, the mechanistic reciprocities between the expression of distinct cadherins have been already suggested, although poorly explored in tumor pathogenesis. Examples include the co-expression of R- and E-cadherin that suppress the progression and metastasis in salivary adenoid cystic carcinomas (49), the combined expression of cadherin-6 and cadherin-11 associated with lymph node metastasis and poor prognosis in oral squamous cell carcinoma (50), and the co-expression of E- and P-cadherin which associated to poor prognosis in breast cancer (23). In particular, further studies of the mechanisms involving E- and P- co-expression led to the proposal of P-cadherin as a marker of an intermediate EMT phenotype (51).

Hence, we expanded our analysis and evaluated tumor characteristics considering the simultaneous expression of the three classical cadherins. This allowed us to not only identify distinct tumor subgroups considering clinico-imaging and neuropathological features, as, more importantly, to confirm the importance of the cadherin interplay in the biological outcome of GBM. For example, the isolated expression of N-cadherin was able to identify the most common GBM profiles (up to 43.8% of the cases), which are the ones commonly found in the frontal lobe, with a typical glial component and few inflammatory infiltrates. Importantly, when N-cadherin expression is combined with E- or P-cadherin, divergent tumor subtypes are observed: E- and N-cadherin co-expressing tumors are frequently located in the temporal lobe, with cortical involvement and cysts, and frequently exhibit an epithelial component; while GBM positive for P- and N-cadherin frequently displays a mesenchymal component, with frequent immune infiltrates. Finally, survival analysis highlights tumors with E- and N-cadherin co-expression as a subgroup with significantly improved progression-free and overall survival, while P- and N-cadherin subgroup had shorter times to recurrence and worse overall survival. It is also noteworthy that, in our data, the P-cadherin increase in recurrence is always accompanied by N-cadherin expression, while co-expression of E-cadherin and N-cadherin was not observed in any recurrent case. Tumors without N-cadherin expression, but with the expression of epithelial

cadherins, commonly exhibit a distinctive neuropathological and MRI patterns, and collectively are subgroups with a worse prognosis.

Taken together, these results suggest that although N-cadherin is the dominant cadherin in GBM, its function can be strongly modulated by the expression of epithelial cadherins. When associated with E-cadherin, it frequently leads to an epithelial-differentiated tumor with a distinct clinical profile and a survival benefit. However, when combined to P-cadherin expression, it leads to tumors with a predominant mesenchymal differentiation, with reduced time for recurrence and survival. In addition, this is the most frequent subgroup upon tumor recurrence. These results led us to hypothesize that the mesenchymal state in glioblastoma is most likely reflected by the combined expression of P and N-cadherins. In fact, these results were further supported by data leveraged from TCGA, showing a significant association between the mesenchymal transcriptional profile and P- and N-cadherin co-expression.

P-cadherin positive status, in our analysis, clearly marks for the worse prognosis subgroups. In particular, isolated P-cadherin or E- and P-cadherin co-expression were found as independent predictors for worse overall survival, while also displaying an imaging profile suggestive of multifocality and propensity to dissemination. This is in concordance with recent reports which associated P-cadherin expression to GBM aggressiveness, through an increase in cell invasion and migration, as well as a decrease in patient survival (46). In addition, we curiously observe that these tumors often display both mesenchymal or epithelial differentiation, but not a homogeneous differentiation, denoting perhaps the presence of heterogeneous cancer cell populations with increased phenotypic plasticity. Thus, our results suggest that neither E-cadherin, nor N-cadherin, expression are able to counteract the disadvantageous biology brought by P-cadherin expression. An apparent profile with increased cancer cell plasticity and markers for enhanced infiltrative potential seems to be denoted by the expression of epithelial cadherins, either P-cadherin expression or E- and P-cadherin co-expression, which we interpret in the context of the possible acquisition of an hybrid EMT state.

The role of E-cadherin, also an epithelial cadherin, with infrequent expression in GBM, is less clear. Our results show a potential dichotomy in the role of this protein. By one hand, when it was co-expressed with N-cadherin, a subgroup with distinct neuropathological, MRI features and improved survival is identified. On the other hand, tumors with isolated expression of the E-cadherin seem to portend a worse prognosis, although they exhibit a similar neuropathological and imaging profile as E- and N-cadherin expressing tumors. Published reports on epithelioid GBM describe this neuropathological and imaging profile, and typically identify tumors with an aggressive course (52, 53). It is thus possible that, as described for other tumors, the expression of other concomitant cadherins, and hence activation of different cellular programs, and not expression of E-cadherin *per se,* will define tumor overall characteristics and aggressiveness.

Finally, the biology of triple positive and triple negative tumors is equivocal. Triple negative tumors do not relate to specific patient characteristics or prognosis. Triple positive tumors present all three morphological components, although significantly associate to predominance of a mesenchymal component. They also are a frequent subgroup in tumor recurrence. These results point to a closer similarity to the P- and N- subgroup. In accordance with these results, we propose a reformulation of the rigid concept of E- to N-cadherin switch as a marker of EMT activation and tumor aggressiveness in GBM. In fact, we hypothesize that tumor characteristics are more likely dictated by the interplay between the expression of different cadherins and considering the expression of a single cadherin may contribute to equivocal results. N-cadherin tumors are the most frequent in our analysis and reflect the average GBM. Our results propose P-cadherin and N-cadherin interplay as a contributor for the mesenchymal-like state in GBM. The presence of an intermediate, more plastic phenotype, with increased tumor aggressiveness, is most likely reflected by the presence of epithelial cadherins, P-cadherin or E- and P-cadherin co-expression. Lastly, GBM is not an epithelial tumor and is therefore less obvious if an epithelial differentiation would present a protective effect. If so, then such putative protective epithelial state in GBM is clearly reflected by an E- and N-cadherin interaction. Further studies inquiring the mechanisms underlying cadherin interplay are warranted.

In addition, upon validation in other series, our results propose cadherin expression subgroups as a reflection of tumor biology and EMT states. Therefore, they become putative biomarkers to guide clinical decision. These results will become particularly interesting with additional characterization of EMT pathways in GBM and identification of treatment targets.

Conclusions

In this study, we document the expression of N-, E-, and P-cadherin in GBM. Our findings establish that cadherin co-expression is associated with consistent clinical, imaging, and neuropathological differences, ultimately correlating with patient prognosis. Additionally, we challenge the notion that an E- to N-cadherin switch is a definitive marker of an EMT-like program in GBM. Instead, we propose that cadherin co-expression subgroups serve as indicators of distinct differentiation stages within GBM.

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Authors' contributions: CN, ASR and JP conceived and design the structure of the work. CN and RC were involved in the experimental work. NM performed the IHQ. CN and ASR performed the experimental data analysis. RT performed the pathology evaluation and performed a critical data analysis. JR and CF were involved in the discussion of the data and review the final version of the manuscript. CN wrote the manuscript. ASR and JP supervised all the work and final-edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Neuro-Oncology Practice

Clinical Predictors in a series of 313 Glioblastoma patients: Insights and Implications

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Abstract:	Background Glioblastoma (GBM) remains a major challenge in oncology due to its devastating prognosis. While extensive literature identifies age, Karnofsky Performance Status (KPS) and extent of resection as prognostic factors in GBM, the optimal utilization of these characteristics to direct treatment strategies remains indeterminate. This study explores the impact of clinical and imaging factors on patient outcomes, and identifies subgroups with poorer outcome to improve initial prognostication. Methods We performed a comprehensive analysis of 313 patients with GBM, capturing detailed data on demographics, clinical presentation and performance status, VASARI MRI features and treatment strategies. Results

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	Univariate analysis identifies age (p=0.009, PFS and p<0.001, OS), clinical presentation (p=0.001, PFS and p=0.003, OS) and autonomy (p=0.066, PFS and p<0.001, OS) as predictors of patient survival. Likewise, 8 VASARI features on initial MRI associated with survival. We further explored and characterized prognosis-associated subgroups, namely elderly patients and patients with early clinical deterioration (n=97, 31%), in an effort to determine features that could improve clinical decision. Finally, multivariate analysis identified age (p=0.043), presentation with focal deficits (p=0.011) or behavior alterations (p=0.040) as independent predictors of shorter time to recurrence. Age (p=0.002), gait imbalance (p=0.025) together with multifocality (p=0.003), nodular enhancing tumors (p=0.041), bilateral contrast-enhancing lesion (p=0.016) were independent predictors of worse overall survival. Conclusions Treatment decision for GBM patients lacks robust prognostic tools to guide initial surgical strategy. This study underscores the importance of integrating demographic, clinical, and radiological factors to refine survival prognostication and optimize treatment decision for GBM patients.
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Clinical Predictors in a series of 313 Glioblastoma patients: Insights and Implications

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Abstract

Background: Glioblastoma (GBM) remains a major challenge in oncology due to its devastating prognosis. While extensive literature identifies age, Karnofsky Performance Status (KPS) and extent of resection (EOR) as prognostic factors in GBM, the optimal utilization of these characteristics to direct treatment strategies remains indeterminate. This study explores the impact of clinical and imaging factors on patient outcomes, and identifies subgroups with poorer outcome to improve initial prognostication.

Methods: We performed a comprehensive analysis of 313 patients with GBM, capturing detailed data on demographics, clinical presentation and performance status, VASARI MRI features and treatment strategies (surgery, EOR and adjuvant therapies).

Results: Univariate analysis identifies age ($p=0.009$, PFS and $p<0.001$, OS), clinical presentation ($p=0.001$, PFS and $p=0.003$, OS) and autonomy ($p=0.066$, PFS and $p<0.001$, OS) as predictors of patient survival. Likewise, 8 VASARI features on initial MRI associated with survival. We further explored and characterized prognosis-associated subgroups, namely elderly patients and patients with early clinical deterioration (n=97, 31%), in an effort to determine features that could improve clinical decision. Finally, multivariate analysis identified age ($p=0.043$), presentation with focal deficits ($p=0.011$) or behavior alterations ($p=0.040$) as independent predictors of shorter time to recurrence. Age ($p=0.002$), gait imbalance ($p=0.025$) together with multifocality ($p=0.003$), nodular enhancing tumors ($p=0.041$), bilateral contrast-enhancing lesion ($p=0.016$) were independent predictors of worse overall survival.

Conclusions: Treatment decision for GBM patients lacks robust prognostic tools to guide initial surgical strategy. This study underscores the importance of integrating demographic, clinical, and radiological factors to refine survival prognostication and optimize treatment decision for GBM patients.

Keywords: Glioblastoma, clinical predictors, VASARI, KPS, age.

Keypoints:

1. Clinical factors and imaging characteristics are interrelated predictors of survival in GBM.
2. Their combined assessment supports early prognostic stratification and personalized treatment.
3. Early clinical deterioration is a key challenge, emphasizing the need for timely prognostic tools.

Importance of the study:

This study analyzes a retrospective cohort of 313 glioblastoma (GBM) patients, integrating demographic data, clinical presentation, functional status, and preoperative MRI features using the standardized VASARI framework.

In addition to identifying readiy available clinical and imaging characteristics associated with patient prognosis, it further delineates high-risk subgroups, including elderly patients, those undergoing biopsy, and a notably large group (31%) who experienced early clinical deterioration.

The findings contribute meaningfully to the ongoing effort to establish robust, multimodal prognostic tools capable of informing treatment decisions at the time of diagnosis

1. Introduction

Glioblastoma (GBM), the most prevalent malignant primary brain tumor in the adult, presents significant challenges in oncological care. The annual incidence is approximately 3,2 cases per 100.000, and the median age at diagnosis is 65[1]. Despite recent advances in treatment modalities, the prognosis for GBM patients remains poor, denoted by a median overall survival (OS) of approximately 11 months and a 5-year survival rate below 6% [2].

The clinical presentation of patients with GBM is often non-specific and progressive in nature, although sometimes mirrors the functional aspect of the affected brain area as focal neurological deficit. Headache is a frequent and early symptom, and its characteristics vary according to tumor size and growth rate. Seizures occur as an initial symptom in approximately 20% of patients with GBM, with an additional 20% experiencing seizure onset at a later stage in the disease progression [3,4]. Other symptoms may include neuropsychological symptoms, and mental status alteration. Clinical presentation has been suggested to associate with survival [5–7].

In addition to clinical presentation, recent studies have explored the relationship between pre-operative MRI characteristics and survival outcomes in GBM patients using qualitative and quantitative characteristics, as well as radiomics [8,9]. Visually Accessible REMBRANDT Images (VASARI) metrics is a validated approach, and several of its features, such as tumor location, deep white matter invasion and ependymal extension, proportion of necrosis or non-enhancing component, have been correlated to patient prognosis and thus pointed as preoperative predicting factors of prognosis [10–13].

Consistently identified prognostic factors linked to poor survival include advancing age, poor performance status (PS) and limited extent of surgical tumor resection [14–16]. Determining how best to use these prognostic characteristics, alongside other clinical and imaging features, to guide treatment decisions remains an uncertainty. In particular, treatment of the elderly patients poses specific challenges, as they often present with higher rates of medical comorbidities, reduced tolerance to treatment toxicities, and, unequivocally, present an overall worse prognosis [17,18].

Another important issue in Glioblastoma is the early deterioration in the postoperative phase observed in some patients. It has been more commonly associated with negative effects from surgery and no clinical predictors have been proposed [19–21].

The combination of a devastating disease with the potential for serious surgical hazards, with serious deleterious effect on patient autonomy and quality of life, urges the identification of characteristics which can improve preoperative prognosis stratification.

In this retrospective study, we meticulously analyze patient demographics, clinical and MRI characteristics, surgical approaches, and outcomes, aiming to elucidate prognostic information among established risk factors. Additionally, we explored subgroups less frequently delineated in existing literature, thereby addressing the complexity encountered in treatment decisions. Our study thus aims to provide insights into the multifaceted aspects of glioblastoma patients.

2. Methods

2.1. Patient selection, demographic and clinical characteristics

A surgical cohort of patients diagnosed with glioblastoma in Hospital de Santo António - Unidade Local de Saúde de Santo António, from January 2010 until December 2017 was reviewed. Exclusion criteria included age inferior to 18 years-old and infratentorial tumors. In addition, all specimens were reviewed and those not compatible with glioblastoma or sparse glioblastoma specimens mostly composed of necrosis or tumor-invaded parenchyma were also excluded **(Supplementary Figure 1)**. A total of 313 patients were included. Of these, 135 (43,13%) had available *IDH* mutation data and a positive mutation rate of 3%. Date of last follow-up was August of 2021.

Clinical records were reviewed in order to document patient demographics, presenting symptoms and functional status upon presentation, after surgery and at 12 months. Preoperative MRI were reviewed and qualitative and quantitative characteristics according to Visually Accessible Rembrandt Images (VASARI) features were documented and validated by an experienced neuroradiologist. Treatment decision (surgery and

adjuvant treatments) and patient follow-up was documented with detail, as well as progression-free and overall survival times.

2.2. Treatment decision

The decision to pursue surgical resection versus needle biopsy was determined either by a consensus between various neurosurgeons or by a multidisciplinary team for patients with worse functional status, and was based in patient traits, autonomy and MRI characteristics **(Supplementary Table 1)**. Use of adjuvant treatments was based on the recommendations of a multidisciplinary team dedicated to neuro-oncology. Post-operative MRI was available for 54 patients and extent of resection was calculated. Resection was codified as: gross-total (GTR) when over 98% of enhancing-tumor was removed, subtotal resection (STR) in over 80% and partial resection (PR) when extent of resection was inferior to 80%[22].

2.3. Statistics

The chi-square test was used to assess group differences, as appropriate. Survival as a function after surgical resection or biopsy was expressed as estimated Kaplan-Meier plots and differences assessed via log-rank analysis. The univariate Cox proportional hazards model was used to determine hazard ratios of each variable as a predictor of OS and PFS. Variables with a p-value <0.05 in the univariable analysis was selected for multivariable Cox regression analysis. A 2-tailed significance level (p-value) of 0.05 was applied.

3. Results

3.1. Patient population and treatment characteristics

Our series comprehends a total of 313 patients with a mean age at diagnosis of 61,743 ± 10,483 years old. We did not observe a trend towards mean age alteration at diagnosis throughout our study timeframe. The youngest patient was 19 years old and the oldest 85 years old. The majority of patients were male (57%, n=178) **(Supplementary Table 2)**.

Functional status at diagnosis was defined using Karnofsky Performance Status (KPS). At presentation, median (IQR) KPS was 80 (70-90). The gross majority (88%) of patients in our series was considered independent at diagnosis (KPS 70-100) **(Supplementary Table 2)**. Surgical removal of the lesion was considered in patients with functional dependency when the autonomy loss was attributed to the mass effect from the lesion or associated perilesional edema **(Supplementary Table 1)**.

Focal neurological deficits were the most common presentation, occurring in 110 patients (35,6%), followed by behavior alterations in 66 patients (21,4%) and headache in 65 patients (21%). Over 17% of patients presented with seizures and in 4 patients the tumor was diagnosed incidentally during imaging exams performed due to trauma or unrelated conditions **(Supplementary Table 2)**.

Average major axis was 6,18 ± 2,09 cm and minor axis 3,8 ± 1,9 cm in our series. The majority of tumors were located in the frontal (n=112, 36,1%) and temporal (n=95, 30,4%) lobes. Seventeen tumors (4,8%) were not located in cerebral hemispheres and were codified as deep lesions, considering their thalamic or basal ganglia location. Up to a fifth of tumors (n=71, 22,7%) were multilobar at presentation **(Supplementary Table 2)**.

In our series we identified 76 tumors as multifocal tumors (24,3%), 89 tumors had ependymal extension (28,4%), 92 tumors had corpus callosum extension (29,4%), including 38 butterfly GBM (12,1%). Cysts were present in 49 tumors (15,7%) **(Supplementary Table 2)**.

Brain tumor biopsy alone was performed in 136 patients (43.5%) and surgical resection in 177 patients (56.5%). Characteristics associated with biopsy decision are detailed in **Supplementary Table 1**. Total resection was achieved in 44,4%, subtotal resection in 40,7% and partial resection in 14,8% of patients **(Supplementary Figure 2D)**. Complications were observed in a total of 19 patients (6%).

Postoperative adjuvant treatment relied in the standard of care, chemotherapy (temozolomide) and radiotherapy (RT) according to Stupp protocol for the majority of the patients (189 patients, 60,4%). Other less frequent treatment options in our series included sequential radiotherapy followed by chemotherapy with temozolomide in 8 patients (2,5%) and radiation alone in 19 patients (6,1%) **(Supplementary Table 2)**.

On the date of the last follow-up, 297 patients had recurred (94,9%). Repeated resection upon first recurrence was performed in 38 patients (12,8%) and in 6 patients (2%) upon second recurrence. Second-line treatments were initiated in 159 patients (53,5%) **(Supplementary Table 2)**.

3.2. Long term outcomes

At last follow-up, 297 patients had recurred and 296 patients had died. Median progression-free survival (PFS) was 4 months and median overall survival (OS) in our series was 10 months. Surgical resection significantly improved progression-free and overall survival to 6 months and 14 months, respectively **(Supplementary Figure 2B-2C).** Likewise, extent of resection significantly stratified patient survival times, with median overall survival of 22 months for patients with total resection **(Supplementary Figure 2E-2F).** Patients who underwent surgical resection and adjuvant treatment with Stupp protocol (n= 138, 44%) had 7 months and 16 months of progression-free and overall survival, respectively. We identified 21 patients (6,7%) with a long-term survival (greater than 36 months) in our series.

3.3. Patient characteristics correlated with prognosis

Univariate analysis of baseline clinical features identified 3 characteristics associated with patient outcome: age, initial performance score and clinical presentation **(Supplementary Table 3)**.

Patient presentation was dictated by various tumor characteristics on initial MRI. For instance, patients with larger tumors more frequently presented with behavior alterations ($p=0.014$), while seizures was the most common presentation for the smaller ones ($p<0.001$) **(Table 1)**. Among the distinct clinical presentations, gait imbalance and seizures appear as indicators of prognosis **(Table 1, Supplementary Figure 3A and 3B).** In particular, patients who presented with gait imbalance experienced the shortest times for recurrence ($p<0.001$, median 2 months) and worse overall survival (median 5 months, $p=0.004$). On the other hand, presentation

with seizures associated with significantly longer PFS (median 8 months, p=0.003) and OS (median 12 months, p=0.006) **(Table 1, Supplementary Figure 3A and 3B)**.

Initial autonomy and functional status also associated to clinical presentation (p<0.001). For instance, patients with poor functional status (KPS <70) were comparatively more likely to present with behavior alterations and gait imbalance **(Table 2)**. Imaging features correlated with worse autonomy status upon diagnosis include deep-seated lesions, multifocality and bilateral contrast-enhancing lesions **(Table 2)**. Similarly to clinical presentation, initial KPS stratified patient overall survival **(Supplementary Figure 3C and 3D, Table 2)** and preserved self-care appears as a key factor for outcome prediction in our cohort (PFS, p=0.042, and OS, p<0.001) **(Supplementary Figure 3E and 3F).** However, it is important to note that initial KPS was a less reliable predictor of outcome in the elderly population **(Supplementary Figure 3G and 3H).**

Finally, age significantly associated with PFS and OS in our univariate analysis and Kaplan-Meier plots **(Table 3, Supplementary Figure 4).** Our series included a total of 140 (44,7%) elderly patients, as defined by age >65 years old. In order to explore an age-relevant cut-off, we considered 4 different older age subgroups: >65 years-old (n=140), >70 years old (n=83), >75 years old (n=35) and >80 years old (n=7) **(Table 3)**. Minor differences were identified in clinical presentation, as focal deficits were significantly more common as a presenting symptom and epilepsy less frequent in older age patients. Despite this, we did not observe differences in initial functional status or initial MRI features **(Table 3)**. With increasing age, patients in our series were gradually less frequently considered for surgical resection **(Table 3)**. In the postoperative period, we document higher rates of autonomy decline in the elderly population, which occurs in over 28% of patients aged >65 (p=0.025) and almost a half of patients aged >80. Adjuvant treatment was also significantly less likely to be initiated in these age groups, particularly in patients >75 and >80 years old. Moreover, we noted this tendency also in the elderly patients submitted to surgery **(Table 3)**. Tumor recurrence rates during adjuvant treatment were significantly higher for patients >65 years old **(Table 3)**.

Survival analysis identified significantly shorter times for recurrence for patients >70 (p=0.037) and >80 (0.027) years old, and a tendency for patients aged >65 (p=0.053). Overall survival was significantly reduced for all age groups (>65, p=0.003; >70 p=0.026; >75, p<0.001 and >80, p<0.001) **(Table 3, Supplementary Figure 4)**. Finally, in our series, 138 patients completed the best standard of care, surgery followed by adjuvant chemotherapy + radiotherapy. Of these, 49 were >65 years old, 22 were >70 years old and 5 were >75 years old. Among patients who underwent surgery followed by adjuvant treatments, no significant differences in

survival were observed, albeit a tendency was observed in PFS for patients >70 years (p=0.069) and in OS for patients >65 years (p=0.069) **(Table 3)**.

3.4. MRI features predictive of prognosis

In the univariate analysis of the VASARI qualitative and quantitative imaging features **(Supplementary Table 4),** we found multiple imaging features that were significantly associated with both OS and PFS. The presence of cysts was the only characteristic identified with an improved prognosis (PFS, p=0.038 and OS, p=0.028). VASARI features that correlated with a worse prognosis, include multifocal disease (PFS, p<0.001; OS, p<0.001), solid/nodular contrast enhancement (OS, p=0.035), pial invasion (OS, p=0.034), ependymal extension (PFS, p=0.036 and OS, p=0.001), deep white matter invasion (OS, p=0.015), bilateral lesion with contrast-enhancement (contrast-enhanced tumor (CET) crosses mid-line) (PFS, p=0.022 and OS, p=0.003), and the presence of satellite lesions (PFS, p=0.021) **(Supplementary Table 4).**

3.5. Clinical and imaging factors associated to early clinical deterioration

Early clinical deterioration, prior to adjuvant treatment initiation, was observed in 97 patients (31%), with a consequent decision to forego further adjuvant treatment. Despite a higher rate of tumor biopsy within this subgroup, these patients remarkably represent 17,5% of the surgical cohort and GTR had been achieved in 8,3% (n=2). We therefore explored separately the biopsy and surgery subgroups with early clinical deterioration **(Table 4)**.

Clinical predictors of early deterioration in patients submitted to biopsy included older age (>70, p=0.04, and >75, p=0.009), presentation with gait imbalance (p=0.007) and incapacity for self-care upon presentation (initial KPS <70, p<0.001). MRI predictive features of early deterioration encompassed multifocality (p<0.001), ependymal extension (p=0.026), bilateral contrast-enhancing lesions (p=0.011), presence of satellite lesions (p=0.007) and mild necrosis (p=0.012).

Among the surgical patients (n=31), no patients traits predicted early clinical deterioration. Preoperative MRI with expansive lesions according to T1/FLAIR ratio (p=0.045), with nodular contrast-enhancement (p<0.001) and mild necrosis (0.046) were significantly more common in this subgroup. As noted by the postoperative

KPS, early deterioration in the surgical group was not attributed to immediate postoperative deterioration **(Table 4).**

3.6. Comprehensive Multivariate Analysis for Outcome Prediction

As described, univariate analysis identifies various clinical and imaging predictors of shorter time to recurrence and overall survival **(Supplementary Table 3 and Supplementary Table 4)**. We confirmed that various of these characteristics predict a subgroup of early clinical deterioration, which occurs with tumor biopsy and surgical resection. Finally, we evaluated these features in a combined (clinical, imaging and treatment features) multivariate model **(Table 5)**. Clinical presentation (focal deficits, p=0.011, and behavior alterations, p=0.040) and age (>75, p=0.043) were identified as clinical predictors for shorter time to recurrence. These 3 characteristics doubled the risk of an earlier recurrence. Similarly, 5 predictors for worse overall survival were identified, independent from treatment characteristics. Specifically, patients older than 75 years old had 2,5-fold (p=0.002) and presentation with gait imbalance had 3,5-fold increased (p=0.025) the risk of death. MRI features that independently predicted a worse overall survival include multifocality (p=0.003) , nodular/solid enhancing (p=0.0041) and bilateral contrast-enhancing lesion (p=0.016) **(Table 5)**.

4. Discussion

Glioblastoma is the most common malignant primary brain tumor and associates with one of the worst 5-year survival rates among all human cancers [23]. The grim prognosis associated with this tumor remains similar despite major developments in diagnostic modalities, surgical techniques and adjuvant treatment strategies [1,24]. Apart from molecular markers, various clinical characteristics have been explored as putative predictors of disease outcome. Nonetheless, there is still significant uncertainty regarding how these predictors should inform clinical decision-making.

Herein, we present the experience of a tertiary hospital with an influence of 591.000 people. All patients included in our series had surgical resection or tumor biopsy with diagnostic intent in order to undergo adjuvant therapies. Our survival statistics are well within the described in the literature [1,24,25], including the survival benefit observed in the subgroup that undergoes surgery followed by adjuvant chemotherapy and radiotherapy and the value of extent of resection [2,14,15,26].

Our results suggest that upon patient evaluation several characteristics are already indicative of distinct patient outcomes. Univariate analysis of initial clinical features evidences that increasing age, presentation with focal deficits, behavior alterations or gait imbalance, as well as low autonomy scores (KPS $\leq$ 70) anticipate a worse PFS and OS.

Similarly, characterization of initial MRI through documentation of VASARI features identified 4 imaging characteristics correlated with poorer progression-free survival (multifocal disease, ependymal extension, bilateral contrast-enhancing lesion and satellite lesions) and 6 features correlated with poorer overall survival (multifocal, thickness of enhancing margin, pial invasion, ependymal extension, deep white matter invasion and bilateral contrast-enhancing lesion). The presence of cysts was the unique characteristic associated with improved patient outcome, which is consistent with data from a recent meta-analysis [27]. The majority of literature regarding initial MRI features focuses on the preoperative differential diagnosis with lymphoma or brain metastasis, as well as tumor grade prediction. Our study is added to a collective effort to stratify initial patient prognosis through preoperative MRI features. To this end, VASARI characteristics are an advantage, as they are easily obtained and highly standardized. Furthermore, we identified unique MRI features linked to early patient deterioration. Other studies have correlated MRI features with patient prognosis and GBM

biology. Features such as deep white matter invasion and bilateral contrast-enhancing lesions relate to the tumor invasiveness capacity, while the quality/thickness of the contrast-enhancing lesion and necrosis quality report to tumor local aggressiveness [28–30]. The presence of satellite lesions or multifocality also probably reflect a more aggressive and advanced tumor, and, especially the latter, have been consistently associated with patient prognosis [31–33].

Taken together, along with the identification of clinical prognosticators, our study reflects on 3 main conclusions.

The reciprocity between MRI features, clinical presentation and initial functional status suggests that these characteristics frequently reflect tumor biology, and should therefore be considered as indirect markers of disease. For instance, poor KPS (<70) more frequently associated with deep-seated lesions, bilateral contrast-enhancing lesions and multifocality, rather than tumor size/major axis or eloquent location. This denotes that low performance scores frequently reflects patient characteristics and tumor intrinsic biology, including its aggressiveness and ability to induce non-focal cerebral dysfunction. Similarly, presentation with seizures is presented here as a good prognosis marker and has been elsewhere strongly associated with tumor biology [5,26]. Thus, these results support that a set of MRI and clinical traits improves surgical decision, especially in particular cases. Further validation of these features in prognostic GBM models and validation in large GBM series is entailed.

Secondly, clinical decision in the elderly should be carefully considered in a case-by-case basis. In our study, evaluation of survival data on this population shows a consistent and significant decrease in the survival statistics, although minimal differences are observed regarding clinical presentation, initial performance status or MRI features. In one hand, we document a higher percentage of biopsies in the elderly patients. We recognize that this result likely reflects the tendency for under-treatment in this population, which is a pertinent issue to be addressed, as there is still ongoing debate regarding the best course of treatment for the elderly patients. Evidence arguing in favor of craniotomy over tumor biopsy in patients older than 65 years old has increased in the last years, and is well documented [34–41]. Recent studies further pointed to a significant impact for EOR in the elderly, as GTR led to longer median PFS and OS when compared to subtotal resection [37,39,42,43][. In addition, published literature shows that although a general worse prognosis is documented for patients older than 65, there is a subgroup of patients with a remarkable favorable prognosis. Frequently, this subgroup underwent GTR, kept a good functional status and proceeded to adjuvant QTRT [37,42]. Our results also identify

a subgroup of older patients with longer survival after surgery and QTRT. On the other hand, it remains unclear how to discern which elderly patients will likely benefit from this course of aggressive treatment. The majority of published studies, as well as ours, report a survival benefit in small subgroups of patients. Data suggesting higher complications rates in the older age exists [44,45] and, most importantly, we show higher levels of autonomy loss in the postoperative period in older age groups. This culminates in a significantly lower percentage of elderly patients initiating adjuvant treatments, even when considering those submitted to tumor surgical resection. Taken together, our data most likely reflect the increase frailty of this subgroup which is frequently unaccounted for in the initial evaluation. The Charlson Comorbidity Index has been evaluated as a predictor of outcome in various series, including some in older GBM patients, with incongruent results [42,46,47]. Importantly, although we have not included patient comorbidities in our analysis, we concur that a collective effort to define a comprehensive geriatric score, or validate published ones, is of utmost clinical relevance [48,49]. We emphasize that while initial functional independency (KPS≥70) is a predictor of better outcome in our series, it did not reach statistical significance when considering the elderly population. This could be explained by the dubious evaluation of patient performance status in older age, as the measure of active work in patients >65 years old becomes more subjective. Considering the nuances of the elderly population, we highlight the importance of thoughtfully consider the presence of MRI features associated with a worse prognosis. In addition, MGMT methylation evaluation before the decision to undergo surgical resection is another idea in these patients, as it readily identifies the group of patients more likely to benefit from adjuvant treatments. These are prominent questions, as finding the delicate balance against ageism but integrating the inevitable increased frailty of elder patients is crucial. In summary, advanced age alone should not preclude surgery followed by adjuvant treatments. However, careful individual evaluation of these patients must consider characteristics that point to an early deterioration, not only as a measure of survival, but also as a reflection of rapid loss of autonomy and, potentially, quality of life. Indeed, due to the retrospective nature of our series we unfortunately do not include quality of life scores in this study and aim to include this outcome in future studies.

Lastly, one of our most striking results, is the percentage of patients who did not initiate adjuvant treatment after diagnosis (n= 97, 31%) due to early clinical deterioration. These include 31 patients that had been submitted to surgical resection which consists of 17,5% of the surgical cohort. Early clinical deterioration is not unique to our series [50]. It importantly reflects the individual variability within the survival statistics for

GBM, as well as the unmet task of refining survival prognostication prior to treatment decision. We characterized this subgroup of patients, identified its clinical and imaging markers, and conclude that these patients are more frequently older patients, with lower performance status and MRI features indicative of tumor infiltration and invasiveness. In patients submitted to surgery, early clinical deterioration was observed more frequently in those which exhibited nodular lesions with avid contrast-enhancement, mild necrosis and mild infiltration/edema (expansive lesions), which may point to tumors with an increased biological growth rate and aggressiveness. Further studies on this topic should aim to confirm MRI features and clinical characteristics, which together with the evaluation of patient frailty, will allow the identification of a subgroup highly likely to experience a poor outcome in the short term. On the meanwhile, it is important to consider that surgical consent and information provided to the families of patients with expected worse prognosis, should include the fact that tumor biopsy and diagnosis do not guarantee nor adjuvant treatment initiation nor an alteration to the natural course of the disease.

Our study is inherently limited by its retrospective design, including the necessity to retrieve data from clinical records. In addition, MRI scans were acquired at various hospitals. It represents a series with an important casuistic of biopsied patients, whose characteristics are in concordance with decision algorithms observed in high-volume centers and previously reported [51,52]. Finally, although IDH status was not evaluated for the whole cohort, it did not influence the analysis in the subset of 135 patients with known IDH status. Despite these limitations, our comprehensive analysis of several clinical and MRI characteristic establishes the possibility of preoperative patience survival prognostication, and hence clinical decision.

We thereby conclude that age, KPS and clinical presentation reflect patient autonomy, frailty and, frequently, underlying tumor characteristics. These clinical characteristics should be complemented with MRI features in order to improve survival prognostication, especially in particular cases.

Ethics

This study has been performed in accordance with the international Helsinki declaration. Moreover, it has also been approved by the ethics committee at Unidade Local de Saúde de Santo António (DEFI 104-17 (093-DEFI/091-CES).

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Authors' contributions

CN conceived and design the structure of the work. CN and CMP collected the data. CN and AF performed the data analysis. CN, CMP and AF wrote de manuscript. RT, AC, CP, CR, JS, MM, JR and ES were involved in the discussion of the data and review the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability

All data generated in this study are presented in this manuscript and supplementary materials or available upon reasonable re- quest from the corresponding author

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Table
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Table 1. Imaging and treatment characteristics across clinical presentation subgroups

	GBM series	Behaviour		Focal deficit		Seizures		Headache		Gait	
	n (%)	**n (%)**	***p-value***	**n (%)**	***p-value***	**n (%)**	***p-value***	**n (%)**	***p-value***	**n (%)**	***p-value***
	313 (100%)	66 (21,4%)		110 (35,6%)		54 (17,5%)		65 (21%)		14 (4,5%)	
MRI features											
Major axis (mean in cm)	6,18 ± 2,09	7,01 ± 3,43	***0.014***	6,53 ± 3,15	*0.249*	4,38 ± 2,46	***<0.001***	6,37 ± 3,36	*0.753*	5,93 ± 3,19	*0.782*
Location		Frontal 40%	*0.230*	Frontal 37% Parietal 27%	*0.092*	Frontal 48%	*0.091*	Temporal 44,6%	***0.014***	Deep 21%	*0.060*
Eloquent	124 (39,6%)	19 (28,8%)	***0.034***	59 (53,6%)	***<0.001***	26 (48,1%)	*0.186*	15 (23,1%)	***0.002***	5 (35,7%)	*0.730*
Multifocal	76 (24,3%)	16 (24,2%)	*0,995*	26 (23,6%)	*0,846*	10 (18,5%)	*0,278*	16 (24,6%)	*0,942*	7 (50%)	***0,022***
Ependymal extension	89 (28,4%)	25 (37,9%)	*0,056*	31 (28,2%)	*0,931*	7 (13%)	***0,005***	22 (33,8%)	*0,281*	3 (21,4%)	*0,550*
Cortical involvement	202 (64,5%)	40 (60,6%)	*0,468*	65 (59,1%)	*0,147*	45 (83,3%)	***0,001***	41 (63,1%)	*0,802*	8 (57,1%)	*0,562*
CET crosses midline	38 (12,1%)	15 (22,7%)	***0.004***	9 (8,2%)	*0.101*	6 (11,1%)	*0.770*	6 (9,2%)	*0.397*	2 (14,3%)	*0.817*
Surgery	177 (56,5%)	37 (56,1%)	*0.836*	58 (52,7%)	*0.242*	33 (61,1%)	*0.523*	42 (64,6%)	*0.175*	7 (50%)	*0.580*
Adjuvant treatment	216 (69%)	41 (62,1%)	*0.174*	73 (66,4%)	*0.458*	43 (79,6%)	*0.064*	50 (76,9%)	*0.122*	5 (35,7%)	***0.006***
median PFS (months)	4	4	*0.640*	3	*0.295*	8	***0.003***	4	*0.938*	2	***<0.001***
median OS (months)	10	8	*0.601*	8	*0.114*	12	***0.006***	10	*0.896*	5	***0.004***

CET= contrast-enhancing tumor

Table
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Table 2. Clinical and treatment characteristics across distinct initial functional status subgroups

	GBM series n (%)	KPS <70 n (%)	KPS ≥ 70 n (%)	*p-value*
	313 (100%)	38 (12%)	275 (88%)	
Presentation				
Behaviour	66 (21,4%)	15 (39,5%)	51 (18,8%)	***0.004***
Focal deficit	110 (35,6%)	10 (26,3%)	100 (36,9%)	*0.202*
Seizures	54 (17,3%)	2 (5,3%)	52 (19,2%)	***0.034***
Gait	14 (4,5%)	4 (10,5%)	10 (3,7%)	*0.058*
Headache	65 (21%)	7 (18,4%)	58 (21,4%)	*0.673*
MRI features				
Major axis (mean in cm)	6,18	6,48 ± 3,55	6, 14 ± 3,23	*0.365*
Eloquent	124 (39,6%)	15 (39,5%)	109 (39,6%)	*0.985*
Deep lesions	15 (4,8%)	6 (15,8%)	9 (3,3%)	***<0.001***
Multifocal	76 (24,3%)	17 (37,8%)	59 (22%)	***0.022***
Ependymal extension	89 (28,4%)	15. (39,5%)	74 (26,9%)	*0.108*
Cysts	49 (15,7%)	8 (21,1%)	33 (12%)	*0.121*
CET crosses midline	38 (12,1%)	10 (22,2%)	28 (10,4%)	***0.025***
Surgery	177 (56,5%)	13 (34,2%)	166 (60,4%)	***0.002***
Adjuvant treatment	216 (69%)	18 (47,4%)	198 (72%)	***0.002***
Recurrence during treatment	141 (64,7%)	13 (72,2%)	128 (64%)	*0.485*
median PFS (months)	4	2	4	*0.276*
median OS (months)	9	5	10	***0.005***

KPS= Karnofsky Performance Status, CET= contrast-enhancing tumor

Table
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Table 3. Comparison of clinical characteristics and survival statistics across different age groups

	GBM series	>65		>70		>75		>80	
	n (%)	n (%)	*p-value*	n (%)	*p-value*	n (%)	*p-value*	n (%)	*p-value*
	313 (100%)	140 (44,7%)		83 (26,5%)		35 (11,2%)		7 (2,24%)	
Presentation									
Behaviour	65 (20,8%)	33 (23,6%)	*0,271*	23 (28,7%)	***0,041***	9 (26,5%)	*0,385*	1 (14,3%)	*0,669*
Focal deficit	116 (37,1%)	65 (46,4%)	***0,002***	31 (38,8%)	*0,717*	16 (47,1%)	*0,201*	3 (42,9%)	*0,748*
Seizures	54 (17,3%)	20 (14,3%)	*0,211*	15 (18,8%)	*0,681*	6 (17,6%)	*0,949*	3 (42,9%)	*0,07*
Headache	74 (23,6%)	21 (15%)	***0,001***	11 (13,8%)	***0,016***	3 (8,8%)	***0,031***	0	*0,137*
KPS preop <70	38 (12,1%)	16 (11,4%)	*0.729*	11 (13,8%)	*0.609*	6 (17,6%)	*0.298*	0	*0.320*
MRI features									
Deep lesions	15 (4,8%)	4 (2,9%)	*0,149*	2 (2,5%)	*0,266*	2 (5,9%)	*0,753*	0	*0,548*
Multifocal	76 (24,3%)	36 (25,7%)	*0,595*	21 (26,2%)	*0,634*	6 (17,6%)	*0,339*	3 (42,9%)	*0,246*
CET crosses midline	38 (12,1%)	14 (10%)	*0.297*	11 (13,8%)	*0.609*	5 (14,7%)	*0.628*	0	*0.320*
Ependymal extension	89 (28,4%)	42 (30%)	*0,581*	23 (28,7%)	*0,942*	9 (26,5%)	*0,788*	2 (28,6%)	*0,994*
Surgery	177 (56,5%)	72 (51,4%)	*0,064*	38 (47,5%)	***0,042***	15 (44,1%)	*0,103*	2 (28,6%)	*0,122*
KPS posop <70	49 (15,7%)	23 (16,4%)	*0,735*	17 (21,2%)	*0,110*	9 (26,5%)	*0,066*	2 (28,6%)	*0,342*
Reduced KPS posop	71 (22,7%)	40 (28,6%)	***0,025***	23 (28,7%)	*0,133*	10 (29,4%)	*0,321*	3 (42,9%)	*0,197*
Adjuvant treatment	216 (69%)	92 (65,7%)	*0,257*	44 (55%)	***0,001***	15 (44,1%)	***<0,001***	2 (28,6%)	***0,022***
Adjuvant treatment after surgery	148 (82,7%)	57 (79,2%)	*0,308*	28 (73,7%)	*0,099*	9 (60%)	***0.015***	1 (50%)	*0.219*
Recurrence during treatment	141 (64,7%)	69 (75%)	***0,006***	32 (72,7%)	*0,211*	13 (86,7%)	*0,065*	2 (100%)	*0,294*
median PFS (months)	4	4	*0.053*	3	***0.037***	2	*0.123*	1	***0.027***
median OS (months)	10	8	***0.003***	6	***0.026***	5	***<0,001***	4	***<0,001***
median PFS with surgery + QTRT (months)	7	6	*0.159*	5	*0.069*	6	*0.618*	-	
median OS with surgery + QTRT protocol (months)	16	14	*0.069*	16	*0.685*	12	*0.179*	-	

KPS= Karnofsky Performance Status, CET= contrast-enhancing tumor

Table
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Table 4. Characteristics of the early clinical deterioration subgroup

	GBM series	Early clinical deterioration - biopsy subgroup		Early clinical deterioration - surgery subgroup	
	n (%)	n (%)	*p-value*	n (%)	*p-value*
	313 (100%)	66 (21%)		31 (9,9%)	
Presentation					
Behaviour	66 (20,8%)	17 (25,8%)	*0.296*	8 (25,8%)	*0.499*
Focal deficit	110 (35,6%)	26 (39,4%)	*0.417*	11 (35,5%)	*0.967*
Seizures	54 (17,3%)	6 (9,1%)	***0.048***	5 (16,1%)	*0.862*
Gait	14 (4,5%)	7 (10,6%)	***0.007***	2 (6,5%)	*0.576*
Headache	65 (21%)	10 (15,2%)	*0.207*	5 (16,1%)	*0.504*
Age > 65	140 (44,7%)	33 (50%)	*0.332*	15 (48,4%)	*0.666*
Age > 70	83 (26,5%)	26 (39,4%)	***0.004***	10 (32,3%)	*0.368*
Age > 75	35 (11,2%)	13 (19,7%)	***0.009***	6 (19,4%)	*0.109*
KPS preop <70	38 (12,1%)	29 (43,9%)	***<0.001***	4 (12,9%)	*0.139*
MRI features					
Major axis (mean in cm)	6,18	6,78	*0,189*	6,496	*0,647*
Eloquent	124 (39,6%)	25 (37,9%)	*0,745*	12 (38,7%)	*0,913*
Deep lesions	15 (4,8%)	5 (7,6%)	*0,235*	2 (6,5%)	*0,650*
Multifocal	76 (24,3%)	35 (53%)	***<0,001***	8 (25,8%)	*0,835*
Ependymal extension	89 (28,4%)	26 (39,4%)	***0,026***	6 (19,4%)	*0,238*
Cysts	49 (15,7%)	3 (4,5%)	***0,020***	2 (6,5%)	*0,248*
Proportion of necrosis >33%	180 (57,5%)	29 (43,9%)	***0,032***	12 (38,7%)	***0,046***
Satellite lesions	76 (24,2%)	35 (53%)	***0,007***	8 (25,8)%	*0,966*
T1/FLAIR ratio - expansive	145 (46,3%)	27 (40,9%)	*0,432*	21 (67,7%)	***0,046***
Thickness of enhancing margin - nodular	60 (19,2%)	15 (22,7%)	*0,722*	9 (29%)	***<0.001***
CET crosses midline	38 (12,1%)	14 (21,2%)	***0,011***	5 (16,1%)	*0.474*
KPS posop <70	49 (15,7%)	25 (37,9%)	***<0,001***	2 (3%)	*0,552*

KPS= Karnofsky Performance Status. CET= contrast-enhancing tumor

Table
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Table 5. Multivariate analysis

		Progression Free Survival				Overall Survival			
		HR	95,0% CI		p-value	HR	95,0% CI		p-value
			Inferior	Superior			Inferior	Superior	
Age	**<75**	1				1		1	
	>75	2,004	*1,022*	*3,932*	**0.043**	2,511	*1,413*	*4,461*	**0.002**
KPS	**90-100**	1				1			
	80	1,295	*0,74*	*2,269*	0.365	1,367	0,846	2,211	0.202
	70	1,054	*0,515*	*2,156*	0.885	1,499	0,825	2,727	0.184
	<70	0,874	*0,366*	*2,088*	0.761	1,389	*0,676*	*2,853*	0.371
Presentation	**Seizures**	1				1			
	Focal	2,563	1,244	*5,28*	**0.011**	1,618	*0,891*	*2,935*	0.114
	Gait	3,783	0,954	*14,998*	0.058	3,587	*1,173*	*10,969*	**0.025**
	Headache	2,758	*1,251*	*6,08*	0,072	1,818	*0,958*	*3,447*	0.067
	Behaviour	2,262	*1,04*	*4,923*	***0.040***	1,673	*0,919*	*3,045*	0.092
Multifocal	**No**	1				1			
	Yes	1,677	*0,919*	*3,061*	0.092	2,189	*1,307*	*3,665*	**0.003**
Thickness of enhancing margin	**Thin**	1				1			
	Thick/Nodular/ Solid	1,081	*0,647*	*1,804*	0.767	2,22	*1,032*	*4,776*	**0.041**
Ependymal extension	**No**	1				1			
	Yes	1,146	*0,656*	*2,004*	*0,633*	1,038	*0,61*	*1,765*	0.891
CET crosses midline	**No**	1				1			
	Yes	1,077	*0,575*	*2,021*	*0.816*	1,72	*1,108*	*2,671*	**0.016**
Surgery	**Surgery**	1				1			
	Biopsy	1,794	*1,077*	*2,989*	**0.025**	2,13	*1,417*	*3,201*	**<0.001**
Treatment	**Yes**	1				1			
	No	13,503	*6,295*	*28,966*	**<0.001**	4,634	*2,939*	*7,309*	**<0.001**

KPS= Karnofsky Performance Status. CET= contrast-enhancing tumor