

Glioblastoma: Treatment Stagnation and Cellular and Molecular Mechanisms

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Abstract

Glioblastoma, a WHO grade IV primary brain tumour, remains as one of the most aggressive forms of human cancer. Despite intensive research efforts into understanding the key drivers of tumour progression, few therapeutic advances have been made, with the current standard of care (the Stupp protocol) remaining unchanged for 15 years. The overall improvement to glioblastoma survival in the real-world population has been attributed to the use of the Stupp protocol, yet evidence suggests that survival outcomes were already significantly improving in the years prior to the introduction of this standard of care questioning the overall veracity of this claim.

Using the Surveillance, Epidemiology and End Results (SEER) registry data we analysed the survival outcomes for real-world glioblastoma patients diagnosed from 2000 – 2016. Our findings show a consistent incremental survival improvement that preceded the introduction of the Stupp protocol and continued to increase at the same rate till 2009, stagnating afterwards. Significantly, however, this survival improvement is short-term for patients, with no survival improvement observed in patients surviving more than 2 years. Additionally, with the exception of complete tumour resection, all treatment modalities did not improve survival beyond 2 years for glioblastoma. These findings highlight the clinical stagnation of glioblastoma treatment and highlight the inability of current treatments to target the underlying causes of tumour progression.

Following the introduction of the Stupp protocol attempts to develop new treatment options have universally been disappointing with a close to 0% success rate for over 1000 phase II and above clinical trials. Conflictingly, many of the therapeutic agents tested have shown promising results in preclinical trials. Current preclinical models, however, test therapies against the primary tumour, which does not recapitulate the biology or targets of tumour

recurrence. We therefore developed a highly sensitive luciferase-based glioblastoma mouse model capable of single cell detection in mouse tissue. Analysis of mouse brain tissue implanted with luciferase-labelled human glioblastoma U87MG or MU20 tumours revealed the presence of tumour cells ubiquitously spread across the supratentorial regions of the brain, and distally located from the primary tumour. These tumour cells were observed as single cells in U87MG implanted mice and clusters in MU20 glioblastoma cells. Remarkably, U87MG tumours did not exhibit invasive margins and were contained within an expansive growth phenotype, suggesting invasion-independent dissemination. Our model is consistent with reports of glioblastoma as a systemic brain disease and is capable of sensitive detection of disseminated tumour cells, a model of recurrence potential. Furthermore, this model can be utilised to investigate new mechanisms of glioblastoma infiltration.

Targeting aberrant angiogenesis in glioblastoma has been the major focus for glioblastoma treatment since the Stupp protocol. Yet after over a decade of basic research and clinical trials, antiangiogenic inhibitors have failed to translate into improved patient outcome. The discovery of abnormalities in the tumour vasculature suggest that there may be alternate mechanisms driving tumour progression. Vasculogenic mimicry has been observed in glioblastoma and presents as a novel aspect of tumour biology, yet the mechanisms and functional relevance of these structures remain unknown. Our study has confirmed the ability of some glioblastoma cell lines to undergo endothelialisation, forming lattice structures similar to endothelial cells when seeded onto Matrigel *in vitro*. One lattice forming cell line, U87MG, was also found incorporate into the tumour vasculature in an *in vivo* orthotopic mouse model. This behaviour was found to be regulated by an expanded TGF- β -ALK1-Smad1/5 signalling pathway. *In vivo* inhibition of the Smad1/5 signalling pathway via intracranial treatment with Ad-Smad6 resulted in reduced endothelialisation in the tumour vasculature and inhibited whole brain infiltration in the U87MG mouse model. Since U87MG

xenograft tumours are non-invasive, these results suggest that endothelialisation may lead to haematogenous dissemination and distal brain infiltration, providing a novel mechanism for glioblastoma progression.

Declaration

This is to certify that:

- 1) The thesis comprises only my original work towards the Doctor of Philosophy, except where indicated in the preface.
- 2) Due acknowledgement has been made in the text to all other material used.
- 3) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Thomas Michael Benjamin Ware

Publications

Thesis-related Articles

Review Article

Ware T, Zhu H-J. Glioblastoma treatment: Where to now? *Integrative Cancer Science and Therapeutics*. 2018;5(3). doi:10.15761/ICST.1000280

Original Article

Chen H, **Ware T**, Iaria J, Zhu H-J. Live Cell Imaging of the TGF- β /Smad3 Signaling Pathway In Vitro and In Vivo Using an Adenovirus Reporter System. *Journal of visualized experiments : JoVE*. 2018;(137). doi:10.3791/57926

Correspondence Article

Ware TM, Zhu H-JJ. USP26 regulates TGF- β signalling by deubiquitinating and stabilizing SMAD7; not applicable in glioblastoma. *EMBO reports*. 2020;21(1):e47030. doi:10.15252/embr.201847030

Other Articles

Original Articles

Ren J, Wang Y, **Ware T**, Iaria J, Dijke PT, Zhu H-J. Reactivation of BMP signaling by suboptimal concentrations of MEK inhibitor and FK506 reduces organ-specific breast cancer metastasis. *Cancer Lett*. 2020;493:41-54. doi:10.1016/j.canlet.2020.07.042

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Kadife E, **Ware T**, Luwor RB, Chan ST, Nurgali K, Senior PV. Effects of EphB4 receptor expression on colorectal cancer cells, tumor growth, vascularization and composition. *Acta oncologica (Stockholm, Sweden)*. 2018;57(8):1043-1056. doi:10.1080/0284186X.2018.1429650

Guo L, Zaysteva O, Nie Z, et al. Defining the essential function of FBP/KSRP proteins: Drosophila Psi interacts with the mediator complex to modulate MYC transcription and tissue growth. *Nucleic acids research*. Published online 2016. doi:10.1093/nar/gkw461

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Stylli SS, Luwor RB, **Ware TM**, Tan F, Kaye AH. Mouse models of glioma. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia*. Published online 2015. doi:10.1016/j.jocn.2014.10.013

Preface

This thesis comprises only my original work towards the Doctor of Philosophy and utilises publicly available databases that have been acknowledged in text. Quoted text on page 4-5 of this thesis has been previously written for an accepted review article publication, Ware *et al.*, (2018).¹ The content of publication was written solely by myself, and both Dr. Hong-Jian Zhu and I contributed to the intellectual concept of the review article.

During my candidature I was the recipient of an Australia Postgraduate Award from the Australian Government, The Nick Christopher Scholarship from the Department of Surgery, Medicine, Radiology and Psychiatry, The University of Melbourne and the Ann Henderson Top-Up Scholarship from Australian Rotary Health in partnership with The Rotary of Templestowe and Dine for a Cure.

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General Introduction

Glioblastoma, a World Health Organisation grade IV primary brain tumour, is one of the most debilitating forms of human cancer resulting in a poor prognosis and low expectancy for quality of life and cognitive function. Despite its rare incidence, glioblastoma is associated with a disproportionate morbidity and mortality and is most common in the seventh decade of life, with rates expected to increase with an aging population. Over 1000 phase II and above clinical trials have been developed to improve patient survival, yet only a few have demonstrated a modest efficacy for newly diagnosed glioblastoma patients. This poor translational success has led to a dire stagnation in survival outcomes for patients. There is a clear discordance between our assumptions and understanding of the key drivers for glioblastoma progression. It is vital that future therapeutic development be based on a more comprehensive understanding of the key mechanisms regulating tumour progression and inevitable patient mortality.

Chapter 1. Literature Review

1.1 Glioblastoma Overview

1.1.1 Glioblastoma Classification

The early classification of glioblastoma was defined from the seminal work of Bailey and Cushing who sought to establish a system that would guide treatment and prognosis through characterisation of the key tumour properties inherent to the underlying biology.² Through extensive examination of brain tumour specimens, Bailey characterised glioblastoma as a heterogeneous and atypical tumour with no resemblance to glial cells. These observations were published in 1928 as part of the first classification system for gliomas, forming the foundation for all modern glioma classification systems. Later in the 1970s the World Health Organisation (WHO) recognised the need for an international classification for gliomas and continued to expand on the foundational work of Bailey and Cushing. Through panelled sessions with the world's foremost prominent neuropathologists and neurosurgeons the first WHO Classification of Central Nervous System Tumours was published in 1979.³ This classification focused on defining entities based on proposed cell origin and morphology and the assignment of malignancy grades related to clinical outcome. In the first edition glioblastoma was designated a grade IV brain tumour but due to a lack of consensus on origin of glioblastoma it was classified under "Poorly Differentiated and Embryonal Tumours". The link between astrocytomas and glioblastoma would not be formally recognised until the next revision in 1993 following evidence of shared molecular characteristics.⁴ In this edition glioblastoma was histologically defined by: *"an anaplastic, highly cellular tumour consisting of fusiform cells, small, poorly differentiated round cells or pleomorphic cells alone or in varying combination. Necrosis, pseudopalisading, fistulous vessels and vascular endothelial proliferation."*, a definition which still remains today.³ In addition to the most common form of glioblastoma the first edition also identified giant cell glioblastoma as a glioblastoma variant, which displayed large atypical glioblastoma cells but prognostically had better

survival outcomes.³ In the third revision published in 2000, gliosarcoma was also recognised as a rare variant of glioblastoma, demarcated by a sarcomatous component and clinically poor survival.⁵ The most recent edition of the WHO classification of CNS Tumours published in 2016 updated the classification to not only reflect histopathological features but also well-established molecular patterns, namely the stratification of glioblastoma into IDH-wildtype (primary glioblastoma) and IDH-mutant (secondary glioblastoma) in recognition of the distinct pathways of genetic development and clinical progression of the disease.⁶ In addition to these molecular updates, a provisional variant of epithelioid glioblastoma was added, which was characterised by a BRAF V600E mutation, joining giant cell glioblastoma and gliosarcoma under the entity of glioblastoma IDH-wildtype.

The establishment of an international glioma classification has undoubtedly impacted upon the choice of treatment and research directions for the glioblastoma. With the ultimate goal of guiding treatment and prognosis, further updates will continually be made to this classification to better align with these outcomes. Precise classification reflecting the clinical history and major molecular drivers of glioblastoma would be considered the holy-grail of glioblastoma classification, providing specific and personalised treatment options.

1.1.2 Epidemiology and Risk Factors

Glioblastoma is the most common malignant brain and other CNS tumour representing 47.7% of all cases, with a reported age-adjusted incidence of 3.21 per 100,000 (normalized to the 2000 US standard population).⁷ It is primarily diagnosed at older ages with a median age of 65 years and has a higher preponderance for caucasian ethnicity and males. IDH-wildtype glioblastoma is the most common form of glioblastoma most often developing *de novo* and represents ~90% of all glioblastoma cases. It arises most commonly in seventh decade of life and most commonly develops within the supratentorial lobes. IDH-mutant glioblastoma occurs in ~10% of all cases and presents most commonly within the fifth decade of life following clinical progression from a lower grade astrocytoma. IDH-mutant glioblastomas

have a preference for developing in the frontal lobe.⁷ Relative survival rates differ significantly based on age category with 1- and 2-year relative survival rates lower in children, 0-14 year, (1yr Relative Survival Rate (RSR) 52.9%; 2 yr RSR 28.8%) compared to Adolescents and Young Adults, 15-39 years, (1yr RSR 74.3%, 2yr RSR 48.0%).⁷ Adults above 40+ years present with the worst relative survival outcomes (1yr RSR 38.1%, 2yr RSR 15.5%).

Besides demographics, high-dose ionizing radiation delivered to the head or neck is a recognised risk factor for glioblastoma though these cases make up a small proportion of total incidence.⁸ No other environmental exposures or lifestyle behaviours has been unequivocally identified as a risk factor. Certain inheritable and spontaneously forming genetic conditions have, however, been associated with increased glioblastoma incidence.⁸ Glioblastoma has been found to occur in 1-3% of patients with Lynch Syndrome, caused by mutations in the mismatch repair genes.⁹ Li-Fraumeni syndrome, associated with a germline mutation in *TP53*, also presents with an increased risk of glioblastoma, most commonly developing in early childhood or after 20 years of age.⁹ Beside these rare inheritable genetic conditions, family history of glioblastoma is rarely observed, and glioblastoma tend to occur sporadically.

Age is considered to be the strongest prognostic factor for glioblastoma with pediatric and adult glioblastoma considered to be of different molecular origin and consequentially have significantly different clinical outcomes.⁷ Considering the clinical and molecular differences for glioblastoma among different age groups, this thesis will focus on adult glioblastoma cases (aged 20+ years).

1.1.3 Clinical Diagnosis

Glioblastoma is initially suspected based on a clinical history of neurological symptoms.

Headaches are the most frequent symptom, observed in over 50% of patients at diagnosis but

seizures, memory loss and cognitive changes also occur in 20-40% of cases.¹⁰ Debilitating symptoms such as hemiparesis, sensory loss, or visual field disturbances are also common, reflecting tumour location.¹⁰ Initial glioblastoma diagnosis is made primarily through brain magnetic resonance imaging (MRI) with and without contrast enhancement.¹⁰ Glioblastoma tumours almost always enhance with gadolinium owing to extensive peritumoural vasogenic oedema that confers to a poorer prognostic outcome.¹¹ The centre of the primary tumour exhibits a heterogeneous hypo- hyper- intensive signal consistent with a necrotic core. Other imaging modalities such as T2/FLAIR can also be used for diagnostic confirmation and determination of infiltrative tumour margins.¹⁰ Furthermore, MR perfusion imaging can be used to assess the vascular properties of the tumour, exhibiting increased relative cerebral blood flow around the peritumoural margins, which carries an inordinate prognostic power for detection of eventual tumour progression and clinical outcome.¹² While most glioblastoma cases present as unifocal lesions on diagnosis, around 20-25% of all tumours are multifocal, consisting of two tumours connected by a diffusive tumour track. Multicentric lesions, where no communicable track is identified between the tumours, has also been reported to occur in 4-6% of glioblastoma cases.^{13,14} These appearances are considered to have arisen from a single tumour, conveying an extensive glioblastoma spread that cannot be appreciated through imaging alone. The mechanism for the development of multicentric tumours is unclear.

1.2 Molecular and Genetic Characteristics

“Early genome-wide profiling of glioblastoma demonstrated a remarkable genomic heterogeneity within the tumour suggesting the existence of molecular subclasses that may clinically impact treatment.^{15,16} The Cancer Genome Atlas (TCGA) project set out to extensively characterize the genomic landscape of glioblastoma and identify the major cancer-causing genomic alterations.^{17,18} The initial publication identified major alterations to the Receptor Tyrosine Kinase (RTK)/RAS/PI3K pathway, in addition to p53 and RB

mutations. EGFR activating mutations or amplification was the most common alteration found to be expressed in 57.4% of glioblastoma cases and has gained much interest as a primary driver of tumour proliferation and survival. Furthermore 50% of glioblastoma tumours with a EGFR amplification, harbor a EGFR variant (EGFRvIII) with in frame deletion of exons2-7 resulting in constitutive activation and enhanced RAS/PI3K signalling.^{19,20} Mutations in *PI3K* (25.1%) and deletions/mutations in *PTEN* (41%) were also commonly found and reported to be mutually exclusive with 59.4% of GBM showing one or the other.¹⁷ These genomic alterations reaffirm a strong association between RTK/RAS/PI3K pathways and tumourigenesis.

The TCGA research network also identified mutations in the p53 pathway, namely amplification of *MDM1/2/4* (15.1%) and homozygous deletion or inactivating mutations in *TP53* (27.9%).¹⁷ In the RB signalling pathway homozygous deletion or inactivating mutations were found in *CDKN2A/CDKN2B* (61%) and *RB1* (7.6%) and amplification of *CDK4/6* (15.5%). Overall signalling alterations were found in RTK/RAS/PI3K signalling in 90% cases, p53 pathways in 86% cases and RB signalling in 79% suggesting a common genetic component to most glioblastoma tumours.

The identification of IDH mutations in glioblastoma provided differentiation between what had until then been only identified as histological primary and secondary glioblastoma.^{21,22} *IDH1* mutations were identified in over 80% of grade II and III gliomas and were conserved during transformation to secondary glioblastoma.²³ In contrast, IDH mutations in primary glioblastoma are rare occurring in under 5% of cases, most associated with younger age and genetic profiles more similar to secondary glioblastoma. IDH mutations are thought to be an early initiator of tumourigenesis and progression to secondary glioblastoma requires further genomic alterations.²⁴ The majority of secondary glioblastoma cases have *IDH1* and *TP53* mutations, whereas primary glioblastoma is most commonly associated with *EGFR* amplification and loss of *PTEN* function.”¹

The identification of consistent abnormalities in known oncogenic pathways found within the TCGA study suggest that patterns of mutation may identify key drivers of glioblastoma progression and may inform future therapeutic decisions. Compilation of the TCGA genetic alterations and gene expression in 840 genes lead to the identification of four unique glioblastoma subtypes; Proneural, Neural, Classical and Mesenchymal, considered to be formed by distinct lineage pathways.^{16,21} The neural subtype, however, could not be determined in subsequent xenograft modelling or in more recent genetic studies, which suggest that the subtype may have been caused by contamination of neural cells.^{21,25} The proneural subtype was associated with younger age, *IDH1* and *TP53* mutations, and *PDGFRA* amplifications/mutations.²¹ This subtype was also found to be enriched for secondary glioblastoma cases, established by prior histologically confirmed lower-grade glioma and *IDH1* mutations. The mesenchymal subtype was defined by a high frequency of *NF1* mutations/deletions and high mRNA expression of *CHI3L1*, *MET* and inflammatory-associated genes. Mesenchymal tumours also presented with increased necrosis, a possible consequence of increased expression of tumour necrosis factor superfamily pathway and NF- κ B signalling. The classical subtype was characterised by *EGFR* amplification, occurring in 95% of all samples, in addition to 93% presenting with chromosome 7 amplification and 10 deletion. There was also homozygous deletion of the *Ink4a/ARF* locus in 95% of samples and a contrasting lack of abnormalities in *TP53*, *NF1*, *PDGFRA*, and *IDH1* genes associated with the mesenchymal and proneural subtypes. Clinically, the proneural subtype was found to have the best prognostic outcome, however, due to the enrichment of *IDH1* mutations identifying with mostly secondary glioblastoma cases, these tumours were found to occur in younger patients and adjustment for age yielded a lower survival outcome for the proneural subtype in comparison to mesenchymal and classical subtypes.²⁶

The TCGA project also identified distinct subsets of DNA hypermethylation in glioblastoma tumours. Promoter DNA methylation sites were enriched in CpG islands, termed glioma CpG

island methylator phenotype (G-CIMP), and associated with the proneural subtype, specifically through a high frequency of *IDH1* mutations.²⁷ Brennan and the TCGA Glioblastoma Analysis Working Group also confirmed the G-CIMP phenotype, identifying an association with *IDH1*, *TP53*, *ATRX* and *MYC* mutations within this subtype.¹⁷ *IDH1* mutations are thought to actively induce to the G-CIMP phenotype through alteration of 2-hydroxyglutarate (2-HG) production. This finding is consistent with the observation that most *IDH1* mutant tumours were G-CIMP⁺, though G-CIMP⁺/*IDH*^{wt} tumours have also been identified.^{17,28,29} The group further expanded the DNA methylation profiles of glioblastoma identifying an additional 5 classes (termed methylation clusters: M1, M2, M3, M4, M6) correlated to; age and survival; somatic mutations in *IDH1*, *TP53* and *ATRX*; the four TCGA gene expression subclasses (mesenchymal, neural, proneural and classical) and 18 selected copy-number alterations.¹⁷ TERT promoter mutations have also been reported in up to 83% of primary glioblastoma and were found to be mutually exclusive of *ATRX* alterations, which were concomitant with *IDH1/2* and *TP53* associated with a proneural subtype.^{17,30,31}

Recently, Behnan and colleagues suggested that the established TCGA glioblastoma subtypes of mesenchymal, proneural and classical could be classified with excellent concordance based on a 12 gene signature set.³² In this classification, proneural was identified by *P2RX7*, *STMN4*, *SOX10* and *ERBB3* expression; classical by *ACSBG1* and *KCNF1*; and mesenchymal by *S100A*, *DAB2*, *TGFβ1*, *THBS1*, *COL1A2* and *COL1A1*.³²

While the glioblastoma subtypes, have been well established and verified through multiple datasets, single cell RNA sequencing analysis reveals remarkable tumour heterogeneity of the transcriptome in *IDH*-wildtype glioblastoma tumours with subtypes variably expressed across individual cells.^{25,33} Furthermore, the main subtype of the primary tumour was found to transition in 45% of recurrent tumours, with the highest change observed in proneural (59% of cases), followed by classical (49% of cases), while mesenchymal was found to be most stable (35% of cases).²⁵ Proneural to mesenchymal transition is the most commonly observed

transition and has been shown to be regulated by the TNF- α /NF- κ B pathways in glioma sphere cultures.³⁴ These results suggest glioblastoma cells maintain a high degree of phenotypic plasticity during glioblastoma development and progression. In further support of this concept, Neftel and colleagues recently established a unified model of cellular states and genetic diversity for glioblastoma.³⁵ Their study characterised four cellular states using single cell RNA-sequencing that recapitulated neural progenitor like (NPC-like), oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like), and mesenchymal-like (MES-like) states. These states associated with genetic alterations in *CDK4*, *PDGFRA*, *EGFR* and *NF1*, respectively and the preponderance of these states were highly consistent with the previously defined three TCGA glioblastoma subtypes. Human glioblastoma tumours were shown to consist of a mixture of these cellular states and in some cases two states were dominant throughout the primary tumour. Orthotopic xenograft implantation of AC-like, NPC-like or MES-like isolated fractions in immunocompromised mice were all shown to be tumourigenic. Following tumour development, analysis of the cellular states revealed a tumour containing all three states in similar distributions demonstrating cellular state transition. Furthermore, it was confirmed that a single cell from a human patient sample, was capable of giving rise to all four cellular states *in vivo*, consistent with glioblastoma plasticity and a steady state baseline distribution of cellular phenotypes. Intriguingly, while cells were able to transition between all states, deletion of chromosome arm 5q containing mesenchymal genes such as *SMAD5* and *TGFBI*, as well as multiple cytokines and chemokines, negatively associated with the MES-like state, suggesting specific signalling pathways are required in order to initiate/progress tumour cell transition.

Further supporting the underlying plasticity of tumour cells, deep whole-genome-sequencing has identified a common pathway of early tumourigenesis, where the characteristic TCGA established driver mutations are acquired by losses or gains of chromosomes up to 7 years prior to diagnosis.³⁶ Initiation of tumourigenesis was reported to start with one or more of

chromosome 7 gain, 9p loss or 10 loss with TERT promoter mutations occurring at a later stage leading to onset of exponential growth. Recurrent tumour cells were found to grow from existing oligoclonal populations in the primary tumour thereby inherently sharing the same genetic features and molecular drivers.

While it is clear that glioblastoma is a dynamic and heterogeneous disease, current diagnostic and treatment methods do not account for these intrinsic cellular differences. The future development of new therapeutics will likely rely on a comprehensive understanding of the complex genetic and molecular makeup of glioblastoma and how it contributes to tumour progression and treatment failure.

1.3 Treatment for newly diagnosed glioblastoma

1.3.1 Surgery

Maximal safe surgical resection remains the current standard of care for glioblastoma.³⁷ Historical surgical resection alone, provided a median overall survival of approximately 3 to 6 months, due to tumour infiltration.^{38,39} Despite the dismal survival outcomes, surgery is an invaluable first line therapy although the optimal extent of tumour resection remains controversial. Retrospective single-centre studies have associated increased extent of resection with better prognostic outcome with some studies suggesting a survival benefit is only observed above 70% to 80% gross tumour resection.^{37,40-44} The proof-of-concept trial for fluorescence-guided resection showed an improved rate of complete resection of contrast enhancing tumours compared to standard techniques and was associated with increased progression-free- but not overall- survival in patients.⁴⁵ Although the volume of residual tumour after surgery negatively correlates with outcome, it has remained impossible to clarify whether extent of resection improves outcome or whether tumours amenable to gross total resection have a different, less malignant course of disease. Maximal tumour resection is favoured, however, due to residual tumour margins exhibiting cerebral oedema or intratumour haemorrhaging, a central cause of morbidity and mortality for patients.³⁷

Advances in surgical techniques, in particular fluorescence guided surgery, have provided a high concordance with extent of resection estimation providing better resections with an increased preponderance towards complete resections.^{45,46} More recently in a retrospective multicentre cohort study, maximal surgical resection of contrast-enhanced glioblastoma was associated with improved overall survival regardless of IDH-status and among patients with IDH-wildtype, independent of DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) status.⁴⁷ Additionally, maximal resection of non-contrast-enhanced glioblastoma also associated with longer overall survival in younger patients irrespective of IDH status.

Surgery, however, carries risks of cerebral oedema and increased intracranial pressure post-surgery. Dexamethasone, a mineralocorticosteroid is commonly administered before and after surgery to prevent cerebral oedema.³⁷ The continued use of dexamethasone for glioblastoma has been the subject of recent controversy, with reports of a possible role in glioblastoma radioresistance and long-term complications with continued use.⁴⁸ Along with its significant side-effects and lack of clarity surrounding its mechanisms of action, rapid de-escalation and alternates to dexamethasone are currently sought.

If surgical resection is not feasible, an image-guided stereotactic serial biopsy is often used for histological confirmation and molecular diagnosis.⁴⁹

1.3.2 Radiotherapy

Radiotherapy has been established as a standard of care for newly diagnosed glioblastoma for the last 60 years.³⁸ Early survival studies at the time demonstrated prolonged survival in the short-term for patients receiving whole brain irradiation of 45 – 50 Gy in small doses over 4 to 5 weeks.³⁸ By 18 months post-operative, however, there was little difference in the overall survival for patients receiving or not receiving irradiation. Nevertheless, median overall survival was found to improve by ~3 months in the irradiated patients conferring a significant

survival advantage and subsequently becoming mainstay treatment for newly diagnosed glioblastoma. A randomised controlled prospective study reported a radiation dose-dependent relationship with survival, with 60 Gy (2 Gy per day during 5 fractions per week) providing the highest median survival outcome of 42 weeks, compared to 36 weeks in the 50 Gy treatment group and 18 weeks in patients that did not receive irradiation.⁵⁰

Advances in radiotherapy technology and techniques have provided more precise delivery of radiation to the tumour site, reducing the adverse events associated with whole brain irradiation, while providing similar short-term survival outcomes.⁵¹⁻⁵³ The use of computerised tomography (CT) and MRI scans in particular have allowed for focal irradiation treatment to the tumour, reducing the incidence of radiation necrosis and intellectual deterioration in long-term survivors, by accurate delineation of the tumour margin.^{51,54,55} The radiological observation that tumour recurrence occurs within 2cm of the original resected mass in 90% of glioblastoma cases has guided the delivery of radiotherapy.^{51,54} There is however, differing opinions among radiologists as to the optimal delivery of 60 Gy irradiation. The European Organisation for Research and Treatment of Cancer (EORTC), recommends 2cm to 3cm margin on T1 enhancement for 60 Gy fractionated delivery, while The Radiation Therapy Oncology Group (RTOG) recommends 2cm on T2 signal abnormality for the initial 46 Gy and 2.5cm margin on T1 enhancement for a 14 Gy irradiation boost. Despite the differences in protocol, among many other variations used within the clinic, smaller margins have not been shown to alter recurrence patterns nor result in inferior survival.⁵⁶ Indeed high dose radiotherapy or dose-escalation (>60 Gy) has not provided superior survival outcomes to 60 Gy standard radiotherapy, although there is an increased incidence of neurological deficits and adverse events associated with increased dosage.

Elderly patients have a higher risk of neurological deficits and decreased quality of life with radiotherapy.⁵⁷ The length of standard treatment and associated morbidity caused by

radiotherapy is problematic given the short median survival outcomes of ~6 months for elderly patients.^{58,59} Additionally, the initial radiation standards for glioblastoma were investigated in a younger cohort (<70 years of age), providing no consensus on the optimal treatment for elderly patients. To establish radiotherapy care for elderly patients, a randomised controlled clinical trial comparing 40 Gy treatment in 15 fractions to standard radiotherapy was conducted which demonstrated noninferiority showing the feasibility of short course hypofractionated radiotherapy.⁵⁸ The Nordic randomised phase III trial confirmed these findings for elderly patients, comparing hypofractionated radiotherapy of 34 Gy in 10 fractions to standard radiotherapy, demonstrating similar survival outcomes between treatment groups.⁵⁹ Current guidelines suggest that the hypofractionated radiotherapy (40 Gy/15 Fr) should be used in elderly glioblastoma (≥ 70 years of age) using the 2 phase RTOG schedule or 1 phase EORTC approach.⁶⁰ Investigations with novel techniques of administering irradiation nor the combination of radiotherapy with candidate radiosensitising agents have shown superior efficacy over standard fractionated radiotherapy.

Radiotherapy treatment has improved in both delivery and specificity through advances in technology and optimisation of standard protocols. The use of MRI in guiding irradiation dosing, along focal external beam irradiation has had the largest impact on improved survival outcomes.⁶¹ Additional studies investigating increased fractionated radiotherapy (>60Gy), however, have not improve clinical outcomes with today's standards.⁶² New techniques such as intensity-modulated radiotherapy (IMRT) and Volumetric-modulated Arc Therapy (VMAT) are becoming increasingly used for glioblastoma treatment, owing to improved delivery of high-dose radiation with improved conformality and less toxicity compared to standard radiotherapy techniques.⁶¹

1.3.3 Chemotherapy

Nitrosoureas compounds were the first major form of chemotherapy explored for glioblastoma due to their lipophilic nature, which was found to have intracerebral activity in

animal glioma models.⁶³ They induce cytotoxicity of tumour cells through inter-strand crosslinks leading to carbamoylation of amino acids, and also cause adduction of N⁷- and O⁶-guanine, which leads to cell-cycle arrest and apoptosis.⁶⁴ The Brain Tumour Study Group (BTSG) conducted a randomised clinical trial enrolling 90% glioblastoma and 10% grade III anaplastic astrocytoma patients to compare combinations of radiotherapy and the nitrosourea agent carmustine (BCNU) following surgical resection.⁶⁵ The median survival for patients receiving only surgery was 14 weeks, compared to 19 weeks in the BCNU-alone group, 36 weeks in the radiotherapy-alone and 35 weeks in the radiotherapy/BCNU concomitantly treated group, suggesting no survival benefit with the addition of chemotherapy. Although overall no statistical difference in survival curves was detected between radiotherapy-alone and radiotherapy/BCNU treated groups the 18-month survival rate was higher in the combined treatment at 10% compared to radiotherapy alone at 4%. A second BTSG randomised clinical trial also reported a survival advantage at 18 months in combined radiotherapy/nitrosourea-based chemotherapy treatment, but there was no significant survival difference with shorter survival time compared to the radiotherapy-alone arm.⁶³ Fine et al., (1993) performed a meta-analysis for postradiation adjuvant chemotherapy from 15 clinical trials and reported a maximal survival increase 11-12% at 18-24 months for glioblastoma patients.⁶⁶ The NCOG randomised phase III clinical trial investigated the efficacy of standard radiation with concomitant oral hydroxyurea followed by adjuvant BCNU or a combination of procarbazine, lomustine (CCNU) and vincristine (PCV). Secondary analysis of stratified glioblastoma patients produced no difference between postradiation BCNU or PCV treatment with a median survival time of 57.4 weeks and 50.4 week respectively.⁶⁷ An early phase II clinical trial evaluating concomitant radiotherapy and bromodeoxyuridine (BrdU) with 1 year adjuvant PCV treatment, also reported a median overall survival of 55.7 weeks.⁶⁸ In a randomised phase III clinical trial, glioblastoma patients were treated with either postradiotherapy PCV or combination difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, and PCV. Although the combination therapy was not superior

median overall survival from surgery was 14.2 and 13.3 months and 8.7 and 6.2% at 2 years respectively.⁶⁹

The Medical Research Council (UK) at the time considered there to be a lack of evidence for the benefit of adjuvant chemotherapy and performed a large scale randomised clinical trial comparing radiotherapy to radiotherapy with adjuvant PCV treatment in high grade gliomas.⁷⁰ This study did not report any improvement in the chemotherapy arm compared to radiotherapy alone for glioblastoma patients (HR, 0.93 [95% CI, 0.77 to 1.12]). Additional randomised phase III clinical trials using adjuvant chemotherapy, also did not show superiority to radiotherapy alone.^{53,71-74} These analyses, however, included both glioblastoma and anaplastic astrocytoma patients in all trials. Furthermore, patient enrolment numbers were low in these clinical trials with fewer than 150 patients enrolled in the largest BTSG trials.

During the time these clinical trials were conducted, there was a consensus that poor overall administration of chemotherapy and a high degree of adverse events limited their overall efficacy for the treatment of glioblastoma. In an attempt to overcome the limitations of systemic administration of chemotherapy, Brem and colleagues investigated the application of a poly-(carboxyphenoxypropane/sebacic acid) anhydride polymer for localised and sustained release of BCNU (BCNU-wafers), which has shown to more effective than systemic administration in a xenograft brain tumour mouse model of gliosarcoma.⁷⁵ BCNU-wafers were subsequently investigated in a phase I/II clinical trial in recurrent gliomas by implanting the wafer in the surgical cavity following reoperation, which deemed the therapy was well tolerated and safe for clinical use.⁷⁶ A randomised, placebo-controlled, phase III clinical trial was further conducted in recurrent gliomas (~65-70% diagnosed glioblastoma) comparing 3.85% BCNU-impregnated wafers or placebo wafers implanted at the time of reoperation.⁷⁷ Median survival was 31 weeks in the BCNU-wafer arm compared to 23 weeks in the placebo arm (HR, 0.67 [95% CI, 0.51 to 0.90]; P=0.006, following adjustment of

prognostic factors). Analysis of glioblastoma patients only also identified a similar survival benefit with a hazard ratio of 0.67 (95% CI, 0.48 to 0.95; P=0.02), following adjustment of prognostic factors, compared to placebo. The unadjusted hazard ratio comparison of BCNU-wafers and placebo arms did not demonstrate significantly different survival outcomes when all glioma cases were considered (HR, 0.83 [95% CI, 0.63 to 1.10]; P=0.19). In a randomised, controlled phase II clinical trial in newly diagnosed high grade glioma 3.85% BCNU impregnated wafers demonstrated survival outcomes in a sub-group analysis of glioblastoma patients, compared to placebo (HR, 0.27 [95% CI, 0.10 to 0.71]; P=0.008).⁷⁸ Patient numbers, however, were small (n=27) due to unavailability of BCNU-wafers part-way through the trial leading to termination of further subject enrolment. Because of the small number of patients used in this clinical trial, a larger randomised, controlled, phase III clinical trial investigating the effects of BCNU-wafers was conducted in newly diagnosed malignant gliomas (~80% diagnosed glioblastoma).⁷⁹ Although the analysis of the overall intention-to-treat cohort demonstrated improved survival outcomes, (HR, 0.73 [95% CI, 0.56 to 0.95]; P=0.0018), a sub-group analysis of glioblastoma patients did not show a statistically significant benefit in the BCNU-wafer arm compared to place (HR, 0.78 [95% CI, 0.60 to 1.03]; P=0.08). Median overall survival was reported as 13.1 months (95% CI, 11.4 to 14.7 months) in the BCNU-wafer treated glioblastoma patients compared to 11.4 months (95% CI, 10.2 to 12.6 months) in the placebo group.

Later, The Gliomas Meta-analysis Trialists (GMT) Group performed a meta-analysis of 12 randomised controlled trials, totalling 1900 glioblastoma patients.⁸⁰ Nitrosoureas were used in all clinical trials, however, combinations or single arm treatments with other chemotherapies were also incorporated into the meta-analysis. Overall, the use of postradiation adjuvant chemotherapy provided improved survival in glioblastoma compared to radiotherapy alone (HR ~0.8; P<0.05), with a small increase in 1-year survival from 35%

to 41% and 2-year survival 9% to 13% respectively. Different radiotherapy regimens and techniques, however, provided uncertainty over the clinical value of this observed effect.

While nitrosourea-based chemotherapy remained the mainstay form of chemotherapy for newly diagnosed glioblastoma in early 2000, another chemotherapeutic agent - temozolomide was being tested in the setting of recurrent glioblastoma. Temozolomide showed penetration of the blood-brain barrier and elicited cytotoxic activity through O⁶-methylation of guanine, triggering cellular apoptosis.⁸¹ In a randomised phase II clinical trial for recurrent glioblastoma, temozolomide was administered orally in 28 day cycles at a dosage of 150 mg/m²/day (750 mg/m² dose per cycle) for patients previously receiving a nitrosourea-based chemotherapy or 200mg/m²/day (1000 mg/m² dose per cycle) for patients with no prior chemotherapy.⁸² There was no significant difference in median overall survival between the temozolomide arm and comparative procarbazine arm, though a statistically significant advantage was conferred at 4-6 months which persisted for 8 months. The primary endpoint, of 6-month progression-free survival (PFS6) with temozolomide was 21%. Another non-comparative phase II clinical trial using the same protocol in recurrent glioblastoma, reported an 8% objective radiological response rate and 43% stable disease at 6 months.⁸³ PFS6 was reported at 18%. Alternate, dose-dense regimes with 150mg/m²/day temozolomide administration on days 1-7 and 15-21 every 4 weeks reported PFS6 at 40-50%.^{84,85} Lower dose prolonged treatment with 75 to 100 mg/m² on days 1 to 21 and repeated on day 29 resulted in a PFS6 of 30%.⁸⁶ Despite the perceived improvement in response rates with these alternate temozolomide schedules, increased or protracted dosing was associated with increased grade III and IV haematotoxicity in these clinical trials. One phase II clinical trial investigated the effects of temozolomide as a neoadjuvant to radiation in newly diagnosed glioblastoma.⁸⁷ Within 28 days post-surgery, temozolomide was given orally at a dose of 200 mg/m²/day for 5 days every 28 days for a maximum of 4 cycles, followed by standard

fractionated radiotherapy. The median overall survival for patients was 13.2 months with a 2-year survival rate of 18%.

Following preclinical evidence of a synergistic effect between temozolomide and radiotherapy *in vitro* and on the basis of clinical trial experience in recurrent glioblastoma, Stupp and colleagues investigated the use of temozolomide as a concomitant and adjuvant to radiotherapy for newly diagnosed glioblastoma.⁸⁸⁻⁹⁰ In a phase II clinical trial, glioblastoma patients receiving partial brain standard radiotherapy were administered concomitant temozolomide (75 mg/m²/day x 7 days/week) for 6-7 weeks, 1 hr prior to radiotherapy or in the early morning on days without radiotherapy. 4 weeks following radiotherapy completion, patients received adjuvant temozolomide chemotherapy (200 mg/m²/day x 5 days, every 28 days for six cycles). Median survival was reported at 16 months (95% CI, 10.9 to 21.2 months), with 1- and 2- year overall survival rates of 58% (95% CI, 47% to 70%) and 31% respectively (95% CI, 19% to 44%). Following these promising results, a phase III clinical trial was conducted comparing radiotherapy-alone to radiotherapy with concomitant and adjuvant temozolomide chemotherapy.⁹¹ The temozolomide doses used in this trial varied slightly with administration of 75 mg/m²/day temozolomide 7 days a week, which continued until the last day of radiotherapy (but no more than 49 days) followed by six cycles of maintenance chemotherapy (150–200 mg/m² x 5 days, every 28 days for six cycles). The final analysis of this clinical trial showed a median overall survival improvement from 14.6 months (95% CI, 13.2 to 16.8 months) in the radiotherapy/temozolomide arm compared to 12.1 months (95% CI, 11.2 to 13.0 months) in the radiotherapy-alone arm (P < 0.001). The 2-year survival rate was 27% (95% CI, 21.2% to 31.7% months) compared to 10% (95% CI, 6.8% to 14.1% months), respectively.

Temozolomide was the first chemotherapeutic to demonstrate an improvement to overall survival for newly diagnosed glioblastoma and remains part of the current standard of care, referred to as the Stupp protocol. A meta-analysis review of temozolomide reports a median

overall survival range of 13.4–19.0 months with the temozolomide-based chemoradiation, compared to 7.7–17.1 months with radiotherapy-alone.⁹²

1.3.4 Therapeutic limitations of the Stupp protocol

The Stupp protocol is the current standard of care for glioblastoma patients consisting of maximum safe surgical resection followed by concomitant temozolomide (75 mg/m²/day for 6 weeks) and radiotherapy (60 Gy in 30 fractions) and then six maintenance cycles of temozolomide (150–200 mg/m²/day for the first 5 days of a 28-day cycle, according to the results of the phase III EORTC-NCIC clinical trial.⁹¹ Although this landmark clinical trial presented a modest improvement in overall survival compared to radiotherapy alone, there are still significant questions regarding the generalisability and optimal scheduling of this standard. One of the most significant considerations for Stupp treatment response was reported in a companion study published alongside the EORTC-NCIC clinical trial.⁹³ MGMT (O⁶-methylguanine-DNA methyltransferase) is a DNA repair gene that removes the alkyl groups from the O⁶ position of guanine, the major target for temozolomide cytotoxic activity.⁹⁴ Epigenetic silencing by MGMT promoter methylation is associated with loss of MGMT expression and is a common occurrence during neoplastic development.⁹⁵⁻⁹⁷ In an analysis of MGMT promoter methylation status in patients enrolled in the EORTC-NCIC, Hegi *et al.*, found MGMT promoter methylation to be an independent favourable prognostic factor.⁹³ Among patients receiving the Stupp protocol, median survival was 21.7 months (95% CI, 17.4 to 30.4 months) with MGMT promoter methylation compared to 12.7 months (95% CI, 11.6 to 14.4 months) without. In the radiotherapy-alone arm median overall survival was 15.3 months (95% CI, 13.0 to 20.9 months) with MGMT promoter methylation and 11.8 months (95% CI, 9.7 to 14.1 months). Improved survival was only observed with the Stupp protocol compared to radiotherapy-alone when patients presented with MGMT promoter methylation (HR, 0.48 [95% CI, 0.31 to 0.75]; P=0.007 log-rank test) (unmethylated patients: HR, 0.69 [95% CI, 0.47 to 1.02]; P=0.06 log-rank test). Despite the

early identification of a lack of efficacy with temozolomide in unmethylated MGMT promoter, MGMT biomarker assessment has not been introduced into clinical decision making.⁹⁸ In subsequent clinical trials ~30% to 45% of patients exhibit MGMT promoter methylation.^{93,99,100} One proposed strategy to improve temozolomide response rates is to inhibit MGMT activity. Previous studies in peripheral blood mononuclear cells suggested that protracted temozolomide exposure could deplete intracellular MGMT levels, thereby increasing temozolomide efficacy.^{101,102} A randomised phase III clinical trial in newly diagnosed glioblastoma was conducted to compare the effect of protracted temozolomide treatment.¹⁰³ Following standard radiochemotherapy patients received adjuvant temozolomide according to the Stupp protocol or dose-dense temozolomide administered at a dose of 75 mg/m² for 21 days every 28 day cycle. Dosing was increased in the dose-dense temozolomide arm to 100 mg/m² providing no grade 3 adverse events were observed. There was no observed difference in median overall survival between the two arms (16.6 months in the standard Stupp protocol and 14.9 months in the dose-dense temozolomide arm [HR, 0.87; P=0.06]), irrespective of MGMT promoter status. In the dose-dense arm methylated MGMT promoter had a median overall survival of 21.9 months vs 15.4 months in the unmethylated patients.

In a further analysis of the EORTC-NCIC Stupp trial, EORTC recursive partitioning analysis (RPA) was determined to be a prognostic marker for Stupp efficacy, with diminishing survival benefits observed as RPA class increased (III to V).¹⁰⁴ With the identification of distinct molecular subtypes in glioblastoma, Brennan and colleagues further examined the prognostic relevance of MGMT promoter methylation, finding that promoter methylation associated with treatment response only in the classical subtype (~25% of tumours at diagnosis), but not mesenchymal or proneural.¹⁷ This finding is further confounded by evidence of subtype switching and a high degree of tumour plasticity contributing to the overall resistance of glioblastoma to current therapies.^{25,35}

After receiving the first-line standard of care, most glioblastoma patients experience disease progression after a median PFS of 7 to 10 months.⁹¹ At this point there is no standard regime and various salvage therapies, or alternative first-line therapies have been tested to improve survival.

1.3.5 Standard of care for elderly patients

Although the Stupp protocol is considered the standard of care, the EORTC-NCIC clinical trial was conducted in relatively young glioblastoma population (median 56 years; max 70 years).⁹¹ The current median age of diagnosis for glioblastoma is 65 years of age and with an aging population, elderly glioblastoma (>65 years of age) is set to become the prevalent population afflicted by this disease.⁷ Management of elderly patients is challenging, however, and clinicians have not adapted the EORTC-NCIC findings directly to elderly glioblastoma therapeutic care, due to the poorer performance status and common comorbidities associated at the time of diagnosis. In particular, exploratory analyses of the EORTC-NCIC reported no benefit with the Stupp protocol among patients aged 61-65 and 66-70 (HR, 0.64 [95% CI, 0.43 to 0.94] P=0.096; HR, 0.78 [95% CI, 0.5 to 1.24] P=0.29, respectively).¹⁰⁵

Radiotherapy-alone therefore was still considered the best standard of care for elderly glioblastoma. In 2011 a phase II clinical trial in the elderly poor performance glioblastoma patients, suggested temozolomide may provide acceptable toxicity with a MGMT promoter methylation associated survival improvement.¹⁰⁶ The NOA-08 randomised phase III clinical trial for high grade glioma (~90% glioblastoma) assessed the efficacy of 100 mg/m² temozolomide-alone on days 1 to 7, 1 week on and 1 week off, versus standard radiotherapy (60 Gy/ 30 Fr) for elderly patients (>65 years of age).¹⁰⁷ Median overall survival was 8.6 months (95% CI, 7.3 to 10.2 months) in the temozolomide arm vs 9.6 months (95% CI, 8.2 to 10.8 months) in the radiotherapy group, demonstrating non-inferiority of treatments. The Nordic randomized phase III clinical trial also assessed treatment in glioblastoma patients 60 years and older, comparing 200mg/m² temozolomide on day 1 to 5, every 28 days for up to 6

cycles vs hypofractionated radiotherapy (34 Gy; 3-4 Gy fractions over 2 weeks) vs standard radiotherapy (60 Gy; 2 Gy fractions over 6 weeks).¹⁰⁸ Median overall survival was longer in the temozolomide arm compared to standard radiotherapy (8.3 months [95% CI, 7.1 to 9.5 months] vs 6.0 months [95% CI, 5.1 to 6.8 months]; HR, 0.70 [95% CI, 0.52 to 0.93]; P=0.01) but not hypofractionated radiotherapy (7.5 months [95% CI, 6.5 to 8.6 months]; HR, 0.82 [95% CI, 0.63 to 1.06]; P=0.12). Patients treated with temozolomide with MGMT promoter methylation had better survival than those with unmethylation. MGMT methylation status, however, did not impact survival in the radiotherapy arms. Above the age of 70, survival was better with temozolomide or hypofractionated radiotherapy compared with standard radiotherapy. Early discontinuation of standard radiotherapy (28% of patients) was common due to risks of morbidity, which could partly explain the lower survival outcomes.

In contrast to the NOA-08 study, the Nordic trial found poor outcomes associated with standard radiotherapy compared to hypofractionated radiotherapy or temozolomide in elderly patients. It should be noted however, that there is a discrepancy in the survival outcomes for patients receiving standard radiotherapy between the two trials (median overall survival: NOA-08, 9.6 months [95%CI, 8.2 to 10.8 months]; Nordic, 6.0 months [95% CI, 5.1 to 6.8 months]).^{107,108} This difference may be caused by the study design, in which the Nordic trial assessed all randomised patients, while the NOA-08 trial excluded patients who were unable to start therapy following randomisation.

The EORTC-NCIC CTG randomised phase III clinical trial combined best of practice methods to assess a new chemoradiation strategy for elderly glioblastoma (≥ 65 years of age).¹⁰⁹ Patients were assigned to hypofractionated radiotherapy-alone (40 Gy / 15 Fr) or hypofractionated radiotherapy with concomitant and adjuvant temozolomide (75mg/m²/day with radiotherapy; 150-200mg/m²/day for 5 days of a 28 day cycle, up to 12 cycles postradiation). Median overall survival was longer with radiotherapy plus temozolomide than radiotherapy-alone (9.3 months [95% CI, 8.3 to 10.3 months] vs 7.6 months [95% CI, 7.0 to

8.4 months], HR, 0.67 [95% CI, 0.56 to 0.80]; $P < 0.001$). In MGMT promoter methylated patients, median survival was 13.5 months with radiotherapy plus temozolomide vs 7.7 months with radiotherapy-alone (HR, 0.53 [95% CI, 0.38 to 0.73]; $P < 0.001$). Survival in unmethylated MGMT patients, however, was not significantly different between radiotherapy plus temozolomide and radiotherapy-alone (10.0 months vs 7.9 months; HR, 0.75 [95% CI, 0.56 to 1.01] $P = 0.055$).

Treatment for elderly glioblastoma remains a difficult consideration owing to the frailty of patients. Current guidelines recommend that patients aged 65 years and above with good performance status, resection and MGMT promoter methylation should receive short-course hypofractionated radiotherapy with concurrent and adjuvant temozolomide. If patients are frail, hypofractionated radiotherapy or temozolomide-alone may be administered depending on MGMT status, however poor performance status is also considered for best supportive care.¹¹⁰

1.3.6 The impact of the current standard of care in unselected real-world population

Although temozolomide demonstrated limited efficacy in a selected subset of patients through clinical trials, its impact on unselected real-world patients has been difficult to determine. Following the introduction of the Stupp protocol, it has been recommended that all patients with good performance be treated with first-line temozolomide therapy.⁹¹ Consequently, it has not been possible to determine the direct impact of temozolomide on the general population, as patients not receiving temozolomide tend to be older with poor performance status and therefore lower survival.¹¹¹ In an attempt to overcome these limitations some retrospective observational studies have compared standard of care effects by era, namely the periods before and after the introduction of temozolomide.^{112,113} These studies report median survival outcomes of ~10-14 months in the temozolomide-era (post-2005) compared to ~8 months in the pre-temozolomide-era. While these results may be consistent with the improved survival trends observed in the temozolomide clinical trials,

caution should be taken when attributing the survival outcomes solely to the introduction of temozolomide chemotherapy. Analysis of time periods preceding the introduction of temozolomide also demonstrate improved survival outcomes over time, suggesting that temozolomide-independent factors have contributed to the improved survival rate.^{114,115} Furthermore, the median overall survival improvement reported in the EORTC-NCIC Stupp clinical trial is small at 2.5 months, leading many to suggest that the success of temozolomide chemotherapy lies in the observed proportional increase in 2-year and 5-year survivors. Survival analysis using US registry data from The Surveillance Epidemiology End Results (SEER) program, however, portrays a more complex trend in glioblastoma survival, with the greatest increase in 2-year survival rates occurring between 2000 to 2005.¹¹⁵ A recent registry analysis using the National Cancer Database (NCDB) also did not report any change to 5-year survival rates from 2004 to 2009, remaining steady around 5% (Odds ratio (OR), 1.001 [95% CI, 0.967 to 1.036]; P=0.97).¹¹⁶ Interestingly, the use radiotherapy but not chemotherapy was associated with increased likelihood of reaching 5-year or more survival (OR, 1.643 [95% CI, 1.386 to 1.948]; P<0.001; OR, 1.122 [95% CI, 0.991 to 1.271]; P=0.07, respectively).

These conflicting results suggest that the use of the Stupp protocol, and temozolomide, has not fulfilled the potential observed in clinical trials. The survival disparities reported in registry real-world data in comparison to clinical trials suggest a variable and individualized response to the current standard of care. In addition to well established prognostic factors such as age, performance status and MGMT promoter status; race, health-care access and enrollment in clinical trials have been closely associated with patient survival.¹¹⁷⁻¹²⁰ Given the often-poorer baseline characteristics of unselected patients and high levels of unmethylated MGMT promoter, the overall impact of the Stupp protocol in the general population may be negligible. Indeed Roger Stupp, the pioneer of the Stupp protocol, and Monika Hegi, who identified the relevance of MGMT promoter methylation with this

protocol, both recognise that there is no evidence to support the use of temozolomide in unmethylated MGMT promoter cases and advocate for omission of chemotherapy in these patients.⁹⁸

Registry data has not strongly supported improved survival outcomes due to the introduction of temozolomide chemotherapy as part of the standard of care. The extensive use of temozolomide has been due to a lack of alternate treatments available, however, clinical trial evidence suggests it benefits a small subpopulation providing little to no benefit for most real-world cases. The relevance of the current standard of care for future therapeutic development is not yet clear.

1.4 Clinical trial development for glioblastoma

1.4.1 Erlotinib

The overall poor prognosis and inherent resistance to therapies resulting in rapid glioblastoma recurrence, lead to the development of targeted therapies for glioblastoma, beyond the non-specific targeting of chemotherapy used extensively in the 1980s to early 2000s. *EGFR* amplification was one of the first and most common alterations determined for glioblastoma and was shown to drive increased tumour aggression and survival in *in vitro* and *in vivo* studies.^{121,122} Preclinically, EGFR activation was shown to contribute to radiation resistance, with inhibition of EGFR leading to radiation-sensitivity of U87MG primary tumours.¹²³⁻¹²⁵ These promising preclinical results lead to the single arm phase I/II clinical trial using erlotinib, a selective inhibitor of EGFR tyrosine kinase activity, in newly diagnosed glioblastoma.¹²⁶ In this study, glioblastoma patients were treated with 150 mg daily erlotinib starting 1 week prior concomitant radiotherapy and temozolomide, with erlotinib treatment continuing with adjuvant temozolomide according to the Stupp protocol. Median overall survival was 15.3 months, which was deemed unsatisfactory compared to the EORTC-NCIC Stupp trial report of 14.6 months median survival a few years earlier.^{91,126} During the same time, another single arm phase II clinical trial for recurrent glioblastoma using carboplatin

and erlotinib combinational treatment was conducted, with unappealing response rates and a median overall survival of 30 weeks.¹²⁷ In a newly diagnosed glioblastoma single-arm phase II clinical trial using daily erlotinib concurrent with the Stupp protocol, a 19.3 median overall survival was reported.¹²⁸ Despite this relatively high median overall survival, no other study has shown any convincing evidence of a survival benefit with erlotinib in newly diagnosed or recurrent glioblastoma.^{129,130} Only one randomised comparative phase II clinical trial was performed comparing erlotinib to temozolomide or BCNU in recurrent glioblastoma.¹³¹ In this study erlotinib failed to demonstrate sufficient single agent activity and failed to be associated with survival with consideration of potential biomarkers, including EGFR amplification and expression. Based on these results, erlotinib did not progress to phase III clinical trials. Other EGFR inhibitors have also been generated and clinically trialled in glioblastoma but have also provided disappointing results.¹³²

1.4.2 Temsirolimus

A significant percentage of primary glioblastoma tumours have *PTEN* gene suppression leading to increased PI3K/Akt pathway activation and downstream mammalian target of rapamycin (mTOR).¹³³ Preclinical evidence using xenograft mouse models demonstrated that mTOR blockage resulted in apoptosis of glioblastoma tumour cells, while also sensitising glioblastoma tumours to radiation.¹³⁴ The chemotherapy agent CCI-779 (temsirolimus) binds to FKBP-12, forming a complex that interacts with mTOR resulting in cell-cycle arrest.¹³⁵⁻¹³⁷ Temsirolimus was initially tested in two single-arm phase II clinical trials for recurrent glioblastoma, administered weekly at a dose of 170 to 250 mg.^{138,139} Treatment efficacy was low with reported PFS6 of 2.3% and 7.8%. One of the clinical trials also reported a median overall survival of 4.4 months (95% CI, 3.6 to 4.8 months).¹³⁹ Combinational therapy with erlotinib or sorafenib with temsirolimus for recurrent glioblastoma, resulted in unacceptably high toxicity limiting with no objective antitumour efficacy.¹⁴⁰⁻¹⁴² A randomised comparative phase II clinical trial was conducted comparing radiotherapy with concomitant temsirolimus

and standard radiochemotherapy with temozolomide in newly diagnosed glioblastoma with unmethylated MGMT promoter.¹⁴³ Temozolomide was administered weekly at 25 mg starting with radiotherapy and continued till tumour progression. Temozolomide and radiotherapy were non-superior to standard radiochemotherapy (HR, 1.16 [95% CI, 0.77 to 1.76]) with median overall survival of 14.78 months (95% CI, 13.27 to 16.39 months) and 16.03 months (95% CI, 13.83 to 18.20 months) respectively.

1.4.3 Vorinostat

Histone deacetylases (HDACs) were identified as important epigenetic modulators in a range of solid carcinomas, with inhibition of HDACs inducing tumour cell differentiation, growth arrest and apoptotic cell death.¹⁴⁴⁻¹⁴⁷ HDAC inhibition was considered to be a novel therapeutic target for glioblastoma, supported by preclinical evidence using the HDAC inhibitor vorinostat, which showed reduced proliferation and tumour growth *in vitro* and in xenograft mouse models.¹⁴⁸⁻¹⁵⁰ Vorinostat was first used in a single-arm phase II clinical trial for recurrent glioblastoma, using 200 mg dosing twice daily for 14 days followed by a 7 day rest period.¹⁵¹ Median overall survival from commencement of treatment was low at 5.7 months with confirmed on-target effects. Despite this poor clinical outcome, the efficacy of vorinostat was tested in a single-arm phase I/II clinical trial for newly diagnosed glioblastoma.¹⁵² Vorinostat dosing was given at 300 or 400 mg/day on days 1-5 weekly during temozolomide chemoradiation. Following a 4- to 6-week rest, patients received vorinostat 400 mg/day on days 1-7 and 15-21 of each 28-day cycle with adjuvant temozolomide. The median overall survival was 16.1 months (95% CI, 13.8 to 20.1 months), which did not achieve the primary efficacy endpoint. Vorinostat has not been developed for a phase III clinical trial but has been trialled as a combinational therapy with other anticancer drugs in phase II studies.¹⁵³⁻¹⁵⁵ The outcome of these studies, however, did not support the use of vorinostat for glioblastoma treatment.

1.4.4 Bevacizumab and Cilengitide

The tumour vasculature has been a major glioblastoma clinical target for over two decades. As early as 1972, Judah Folkman hypothesised the relationship between angiogenesis and glioblastoma pathology reporting that glioblastoma tumours contained the highest levels of microscopic angiogenesis when compared to other solid malignancies including sarcomas, adenocarcinomas and low grade gliomas.^{156,157} In vascular cancers, angiogenesis pathways are upregulated activating endothelial cells, which results in characteristic loss of polarity and abluminal sprouting.¹⁵⁸ These activated endothelial cells protrude into the tumour mass forming abnormal blood vessels that are leaky, dilated and contain large fenestrations, contributing to the oedema radiologically observed in glioblastoma.¹⁵⁹

Vascular endothelial growth factor, a major driver of angiogenesis, is found upregulated and highly expressed in glioblastoma tumours and is associated with a poor prognosis.¹⁶⁰⁻¹⁶²

Additionally, its receptor VEGF receptor 1 (VEGFR1) is also upregulated in endothelial cells of the tumour vasculature.^{160,162} Inhibition of VEGF in xenograft mouse models result in reduced growth of the primary glioblastoma tumour.^{163,164} Early clinical trials in other solid malignancies, however, suggested limited benefit from antiangiogenic drugs when used as single agents, relative to measured survival benefit.¹⁶⁵ In contrast, antiangiogenic inhibitors combined with chemotherapy had demonstrated increased survival outcomes in previously treated or untreated metastatic colorectal cancer.^{166,167} These findings lead to the development of a small non-comparative phase II clinical trial to investigate the efficacy of bevacizumab, a monoclonal antibody against VEGF, in combination with irinotecan for recurrent glioblastoma.¹⁶⁸ 15 mg/kg bevacizumab was administered every 21 days with irinotecan on days 1, 8, 22, and 29. PFS6 was 46% (95% CI, 32% to 66%) and median overall survival was 42 weeks (95% CI, 35 to 60 weeks). Objective response rates were observed in 57% of patients. In a follow-up study, tumour specimens collected at the time of diagnosis from patients that were subsequently treated with bevacizumab and irinotecan were assessed for

VEGF expression via immunohistochemical.¹⁶⁹ The study found that high VEGF expression associated with improved radiographic responses, however, this did not associate with survival benefit.¹⁶⁹ A larger phase II noncomparative clinical trial was further conducted to test the efficacy of bevacizumab alone and in combination with irinotecan in recurrent glioblastoma.¹⁷⁰ PFS6 and median overall survival was 42.6% and 9.2 months in the bevacizumab-alone arm and 50.3% and 8.7 months in the combinational treatment.

In the same year another phase II clinical trial investigating the efficacy of single-agent bevacizumab followed by combined bevacizumab and irinotecan in recurrent glioblastoma was published. PFS6 was reported at 29% with objective radiological responses observed in 35% of patients.¹⁷¹ Median overall survival was 31 weeks. The results of this clinical trial also highlighted issues with the current Macdonald criteria for radiological responses for therapeutic efficacy. It was observed that most patients exhibited an initial radiographic response, resulting in a high objective response rate, however, nearly half the initial responders had rapid subsequent progression, suggesting a lack of specific effect on tumour cell biology.^{171,172} These inconsistencies lead to the development of a new radiological response assessment for therapeutic efficacy developed by the Response Assessment in Neuro-oncology Working Group, also known as the RANO criteria.¹⁷³ Despite, no clear overall survival benefit in recurrent glioblastoma, bevacizumab received FDA approval in 2009 based on radiological responses, perceived prolongation of PFS and an overall lowering of glucocorticoid steroids to control tumour-related oedema.

A phase II single arm clinical trial in newly diagnosed glioblastoma was conducted to investigate efficacy of combined 10 mg/kg biweekly bevacizumab plus standard temozolomide during and after radiotherapy.¹⁷⁴ Overall survival was 19.6 months (95%, 16.1 to 23.3 months), which was not significantly different to a their control comparison study of 21.1 months (95% CI, 18.9 to 25.2 months), which used the Stupp protocol, with some patients receiving salvage bevacizumab. Another similar phase II clinical trial using

combined bevacizumab with standard radiochemotherapy followed by bevacizumab, standard temozolomide and irinotecan for newly diagnosed glioblastoma also reported similar survival median overall survival outcomes of 21.2 months (95% CI, 17.2 to 25.4 months).¹⁷⁵ Results of these phase II clinical trials supported the development of two randomised, placebo-controlled phase III clinical trials for newly diagnosed glioblastoma.^{99,176} In both trials, bevacizumab was administered at 10 mg/kg fortnightly concurrent with radiochemotherapy and adjuvant temozolomide. Bevacizumab was continued until tumour progression in one trial, while the other increased bevacizumab dosing to 15 mg/kg following completion of adjuvant temozolomide which continued every 3 weeks until tumour progression. No improvement in overall survival was observed in either trial compared to placebo. Continuous unchanged bevacizumab treatment yielded a median survival of 15.7 months (95% CI, 14.2 to 16.8 months) compared to 16.1 months in placebo (95% CI, 14.8 to 18.7 months).⁹⁹ Dose intensified bevacizumab also did not produce a significant difference in survival with median overall survival of 16.8 months with bevacizumab and 16.7 with placebo treatment (95% CIs not reported) (HR, 0.88; P=0.10).¹⁷⁶ In a follow-up analysis of the phase III AVAglio study, health-related quality of life (HRQoL) reported by self-assessment, was similar between bevacizumab and placebo treated patients, which was maintained during the progression-free time, however, bevacizumab treated patients exhibited a longer progression-free time longer stabilisation of the HRQoL. These findings, however, did not examine HRQoL after tumour progression with bevacizumab nor do they suggest a direct benefit from bevacizumab since this treatment commonly associated with a de-escalation of corticosteroids use.¹⁷⁷ In order to determine if bevacizumab may provide a benefit in a subpopulation, a randomised phase II clinical trial investigated superiority of bevacizumab plus irinotecan vs temozolomide with radiotherapy in newly diagnosed glioblastoma with nonmethylated MGMT promoter.¹⁷⁸ PFS6 was improved with 42.6% in the temozolomide arm compared to 79.3% in the bevacizumab/irinotecan arm. Overall survival,

however, was not different between the arms (bevacizumab/irinotecan: 16.6 months [95% CI, 15.4 to 18.4 months]; temozolomide: 17.5 months [95% CI, 15.1 to 20.5 months]).

$\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are also heavily expressed in the tumour vasculature of glioblastoma and have been shown to be important regulators of angiogenesis and tumour cell aggression.¹⁷⁹⁻¹⁸² Cilengitide is a potent and selective inhibitor of $\alpha v\beta 3$ and $\alpha v\beta 5$ activity and was investigated in clinical trials based on antitumour benefit demonstrated in preclinical glioblastoma models.¹⁸³⁻¹⁸⁷ In a randomized phase II clinical trial, cilengitide was tested first in recurrent glioblastoma using two dosing regimes; 500 mg or 2000 mg twice weekly.¹⁸⁸ The highest response rates were reported in the 2000 mg arm with PFS6 at 15% (95% CI, 5.7% to 29.8%) and median overall survival of 9.9 months (95% CI, 6.4 to 15.7 months), though there was no significant difference compared to the lower 500 mg arm. Following the results of this clinical trial, a single-arm phase I/IIa clinical trial in newly diagnosed glioblastoma was conducted using 500 mg cilengitide twice weekly, concurrent with the Stupp protocol.¹⁸⁹ Cilengitide treatment was shown to cause radiation sensitization of glioblastoma cells in a xenograft animal models, suggesting the potential for a synergistic effect clinically.¹⁹⁰ Median overall survival was reported as 16.1 months (95% CI, 13.1 to 23.2 months), with improved median survival reported in MGMT promoter methylated patients compared to unmethylated (HR 0.44, 95%, 0.21 to 0.91; P=0.022).¹⁸⁹ In an attempt to establish an optimal dose for cilengitide, the New Approaches to Brain Tumor Therapy (NABTT) Consortium investigated 500 mg and 2000 mg twice weekly dosing concurrent with the Stupp protocol in newly diagnosed glioblastoma. Median overall survival was reported at 19.7 months (95% CI, 13.3 to 21.6 months) in the 500 mg arm and 20.8 months (95% CI, 17 to 24 months) in the 2000 mg arm. Although the difference was not statistically significant, the authors suggested that 2000 mg should be used as the standard treatment due to similar toxicity and a perceived additional survival benefit. Based on these findings a randomised phase III clinical trial was conducted for newly diagnosed glioblastoma with methylated MGMT promotor.¹⁹¹

Patients were randomized to 2000 mg twice weekly cilengitide with concurrent Stupp protocol or Stupp protocol-alone. Median overall survival was 26.3 months (95% CI, 23.8 to 28.87 months) in the cilengitide arm and 26.3 (95% CI, 23.0 to 34.7 months) in the control arm. In addition to no clinical efficacy in the primary cohort, no clinical subgroup derived any benefit from cilengitide treatment. The findings of this clinical ended the development of cilengitide for glioblastoma treatment.

1.4.5 Combinational therapy with CCNU

Prior to the establishment of the Stupp protocol, a small non-randomised phase II clinical trial (UKT-03) conducted by Herrlinger and colleagues examined the use of radiotherapy concomitant with combined temozolomide and CCNU treatment in newly diagnosed glioblastoma.¹⁹² Concomitant with standard radiotherapy, patients were administered 100 mg/m² CCNU on day 1 followed by 100 mg/m²/day temozolomide on day 2 to 6 up to 6 cycles. Median overall survival was 22.6 months (95% CI, 12.5 to not assessable) and the 2-year survival rate was 44.7%. The results of this trial did not immediately move to a phase III clinical trial due to publication in 2006 following reports of the EORTC-NCIC phase III clinical trial.⁹¹ A long-term follow up of this study, included 8 patients that received an intensified dose of 100 mg/m² and 150 mg/m² using the same protocol as previously described.¹⁹³ After 41.5 months, median overall survival in this cohort had not been reached and was significantly higher to the standard dose (22.6 months; P=0.024). The updated 2-years revival rate was reported at 47.4% and 4-years survival rate at 18.5%. MGMT status was still a significant factor for prognosis (median overall survival methylated 34.3 months compared to unmethylated 12.5 months). Following this, CCNU was trialed in a number of phase II and III clinical trials for efficacy in recurrent glioblastoma, as a single agent and in isolation, though the results of these trials did not support clinical efficacy.^{100,194-198} In 2019, following the lack of overall clinical success for glioblastoma, Herrlinger and colleagues revisited the results of the UKT-03 trial, designing a randomized phase III clinical trial for

newly diagnosed glioblastoma, which compared CCNU/temozolomide therapy to standard temozolomide therapy in methylated MGMT promoter patients.¹⁹⁹ In this study patients were treated with standard Stupp protocol or 100mg/m² CCNU on day 1 of radiotherapy plus temozolomide 100-200mg/m² on days 2 to 6 per cycle, with 6 cycles total. Median overall survival improved from 31.4 months (95% CI, 27.7 to 27.1 months) with the Stupp protocol to 48.1 months (32.6 months to not assessable) with CCNU/temozolomide (HR, 0.60 [95% CI, 0.35 to 1.03]; P=0.0492 by log-rank test). Though this clinical trial demonstrated a significant survival advantage with combined CCNU/temozolomide, treatment groups were small (63 in the Stupp arm and 66 in CCNU/temozolomide arm) limiting the overall translation of these findings into clinical practice. Additionally, the shortening of the temozolomide treatment to 5 days with concurrent radiotherapy in the experimental arm, creates in effect two interventions, one being the application of CCNU and the other being short course temozolomide with radiotherapy. Maturation of the study results will be needed to add further clarification to these findings.

1.4.6 Tumour-Treating Fields

The lack of success with traditional therapies, lead to an interest in alternate treatment modalities. Tumour-treating fields (TTFields) therapy is a novel electric field-based treatment for cancer and has emerged as a therapy of interest for glioblastoma. *In vitro* studies suggest that low-intensity alternating electric fields specifically affect dividing cancer cells (including human glioblastoma cell lines), but not quiescent cells.²⁰⁰ Following stimulation, tumour cells undergoing division exhibit cell proliferation arrest and/or destruction of cells, thought to be due to interference with mitotic spindle formation and the generation of dielectrophoretic forces acting on polar and charged elements of the cell during cleavage.^{200,201} *In vivo*, 2V/cm alternating electric fields applied externally in F-98 orthotopic rat glioblastoma model tumour inhibited tumour growth by 42.6% with two directional fields and 53.4% with three directional fields.²⁰² These results lead to a pilot clinical trial of TTFields in recurrent

glioblastoma.²⁰² 10 patients were treated for an average of 18 hrs per day until progression with fields of 1-2V/cm generated at 200 kHz with fields switched sequentially every 1 sec between two perpendicular directions. PFS6 was 50% weeks and median overall survival 62.2 weeks. In a randomized semi-comparative phase III clinical trial in recurrent glioblastoma, patients received TTFields, generating 200 kHz electric fields (intensity of >0.7 V/cm at the center of the brain), in two perpendicular directions (operating sequentially) or physician's choice chemotherapy.²⁰³ PFS6 and median overall survival was 21.4% and 6.6 months in the TTFields arm and 15.1% and 6.0 months in the active chemotherapy arm (HR for overall survival, 0.86 [95% CI, 0.66 to 1.12]; P=0.27), concluding non-superiority. Combinational TTFields/chemotherapy treatment *in vitro* using dacarbazine (DTIC), an alkylating chemotherapy drug with similar activity to temozolomide, showed superior inhibition of cell proliferation compared to DTIC alone.²⁰⁴ A small pilot study of 10 newly diagnosed glioblastoma patients with combinational TTFields and temozolomide postradiation, reported median PFS of 155 weeks and median overall survival > 39 months (MGMT status was not provided).²⁰⁴ Based on the prospect of a synergistic antitumour effects, a large randomized comparative phase III clinical trial was designed for newly diagnosed glioblastoma.²⁰⁵ Patients were treated with either 200 kHz TTFields plus standard temozolomide or standard temozolomide postradiation. Interim analysis revealed median PFS of 7.1 months (95% CI, 5.9 to 8.2 months) in the TTFields plus temozolomide arm and 4.0 months (95% CI, 3.3 to 5.2 months) in the temozolomide-alone arm. Median overall survival in the intention-to-treat population was 19.6 months (95% CI, 16.6 to 24.2 months) and 16.6 months (95% CI, 13.6 to 19.2 months) respectively (HR, 0.74 [95% CI, 0.56 to 0.98]; P=0.03 log rank test. Long-term follow up of the clinical trial reported 2-, 3- and 4-year survival rates for TTFields plus temozolomide vs temozolomide alone of 43% (95% CI, 38 to 47%) and 30% (95% CI, 24 to 37%), 24% (95% CI, 19 to 29%) and 16% (95% CI, 11 to 23%), 17% (95% CI, 13 to 23%) and 10% (95% CI, 6 to 18%).²⁰⁶ Following these clinical trials TTFields has been FDA approved for use in both newly diagnosed and recurrent glioblastoma. There

are, however, controversies surrounding the reported benefit of TTFields for survival in newly diagnosed glioblastoma.²⁰⁷ The lack of a placebo-controlled arm leads to an inability to blind the study, which creates a challenge for objective observations of the primary endpoint PFS and concordantly leads to a higher level of care in these patients. Additionally, following clinical progression, patients were provided with salvages therapies consisting of nitrosoureas, bevacizumab and further temozolomide, which given the differences observed in PFS may contribute to changes in overall survival. Additionally, not all patients were prescribed standard radiochemotherapy as per the Stupp protocol with the study including BCNU-wafer implanted patients. Significantly there is a large delay between diagnosis and randomization of 3.8 months, in which 82 of 1019 patients had progressed, contributing to a 2 month difference in overall survival between the registered and randomized patients. Patients randomized therefore were likely to have better baseline prognosis and the generalisability of the presented data is difficult to decipher.

1.5 Understanding the failure of clinical trials

1.5.1 Clinical trial development and design

There have been almost 2000 clinical trials developed for glioblastoma worldwide, for which only 13.2% have provided results.²⁰⁸ Most clinical trials are phase II or phase I/II (70.3%), and primarily target recurrent glioblastoma.^{208,209} Significantly, there is a lack of progression from phase II to phase III clinical trials, which total 6.5% of all clinical trials.²⁰⁸ Of these phase III clinical trials, only 4 have published positive results (~3% of all phase III clinical trials); with two comparing combined radiotherapy and temozolomide in adult or elderly glioblastoma; 1 comparing the addition of lomustine to standard chemoradiotherapy in MGMT promoter methylated glioblastoma; 1 comparing NovoTTF with standard chemoradiotherapy.^{91,109,199,206} Median overall survival improvements for these trials were 2.5 months, 1.7 months, 16.7 months and 4.9 months, respectively. Because of controversy in the design of clinical trials for combinational therapy with lomustine and NovoTTF, standard

chemoradiotherapy with temozolomide remains the clinical standard of care for newly diagnosed glioblastoma.¹¹⁰ This overall success rate for glioblastoma trials is low compared to other cancers, where 37% of phase III clinical trials are likely to receive FDA approval.²¹⁰ Concerningly, more than half of all phase III clinical trials proceeded despite negative phase II clinical results or without any prior phase II trial, suggesting a discordance with the scientific development of clinical trials.²¹¹

The majority of glioblastoma phase II clinical trials are non-randomised, non-controlled single arm studies (74% for newly diagnosed, 90% for recurrent glioblastoma).²⁰⁹ While single arm studies have the attraction of being smaller, less expensive and easier to employ, they are associated with an increase of bias and false positive results.²¹² Only 15% of phase II clinical trials use a randomised controlled trial (RCT) design, with the limiting factor being overall sample size (average sample size: RCT, 131; non-RCT, 50). 59% of phase II clinical trials used PFS as a primary endpoint and only 4% confirmed pharmacokinetics/pharmacodynamic of the interventional therapy. 27% of all phase II clinical trials used overall survival as the primary endpoint.

1.5.2 Limitations of primary endpoints

The use of PFS and objective response rates as primary endpoints are problematic for glioblastoma due to the radiological assessment methodology. Prior to 2010, the MacDonald overall radiological response (ORR) was used to determine the reduction of tumour burden with interventional treatment, in particular cytotoxic chemotherapy.²¹³ ORR, however, has significant limitations in that it is unable to provide a consistent and meaningful clinical benefit. There are multiple reasons for this failure, which include open-labelled trial design, an inability to accurately detect the tumour margins (which is further confounded by changes to oedema with use of antiangiogenic inhibitors and corticosteroids) and pseudo-response/progression.²¹⁴ While the latter two scenarios have improved with the development of the RANO criteria, ORR still remains an unreliable surrogate for clinical significance.¹⁷³

PFS is the most used clinical endpoint for phase II trials, though its use in single arm studies is questionable, due to variation in the development and progression of many cancers including glioblastoma.²¹⁵ PFS has the advantage of including death events and reports a measure of treatment response, however, similar to ORR, PFS is based on radiological assessment and therefore is subject to pseudo- response/progression errors, bias from open-label review and most significantly have a poor association with overall survival.²¹⁶

Overall survival remains the gold standard for clinical trial design, however, due to the variation of survival times for glioblastoma, overall survival must be incorporated into a RCT design. Many single arm studies compare overall survival and PFS to historical records, reporting improved survival outcomes. This poor clinical design dismisses the large variation observed between clinical trials and makes the false assumption that survival with the best standard of care remains static over time. Large registry data analysis dismisses the idea of static survival demonstrating a small but significant yearly incremental improvement to glioblastoma survival since the introduction of the Stupp protocol, thereby invalidating this assumption.¹¹⁵ Overall survival as an endpoint does, however, have limitations, in that a placebo arm should be included to diminish the effects that a higher level of care may provide in the experimental arm.¹¹⁷ Additionally, patients will often receive salvage therapy following tumour progression, or discontinuation of therapy, which may impact the overall survival for those patients creating an ethical dilemma for clinical trial design.

1.5.3 Unrepresentative selection of patients

Even with the optimal study endpoint, there are additional issues that need to be considered for glioblastoma clinical trial development. Inclusion criteria for glioblastoma often favours good performance status and a median age of 55.3 years, which is significantly lower than the median age of the population of 65 years.^{7,217} The demographic composition of glioblastoma clinical trials poorly represents the broader population often skewing gender and race/ethnicity in favour of males and caucasian patients.²¹⁷ This demographic trend is most

often observed in industry-supported phase 1/II clinical trials, though phase III clinical trials often enrol a wider diversity compared to earlier phase trials creating further biases and inconsistencies in clinical trial development.^{120,217} The lack of representation in glioblastoma trials, in particular with respect to age, leads to questionable translation of novel treatments into the general population, where efficacy may be low to negligible.

1.5.4 Invalid assumptions for survival analysis

There is evidence to suggest that glioblastoma patients have a nonconstant hazard of death over time, suggesting that the estimates of survival at time of diagnosis may not be relevant to those who survive a longer period of time.^{218,219} Analysis of clinical trial data in the Stupp era have shown that patient survival is lowest within the first 2 years of diagnosis, but the 1-yr conditional survival rates increase and plateau in subsequent years, resulting in higher a higher probability of surviving an additional year for patients already having survived 3 years compared to the overall 1-year survival rate for newly diagnosed glioblastoma.²¹⁸ The differences in long-term survival outcomes appears to reflect a difference in tumour biology compared to the short term survivors. Given these findings the use of Kaplan-Meier and Cox proportional hazards models would not appropriate to analyse survival of these long-term population, which would have reduced study power to detect any meaningful change within this cohort.

1.5.5 Inappropriate preclinical models

Despite a raft of issues with current glioblastoma clinical trials development, the overall negative results highlight inappropriate therapy targets. Many of these targets are tested successfully in preclinical models suggesting a discordance between preclinical responses and overall translation into clinical trials. The poor predictive value of preclinical studies testing therapeutic drugs has been observed in almost all clinical trials since the approval of temozolomide, questioning the validity of our preclinical measurements and whether they truly

represent the clinical target. Current preclinical models used to predict therapeutic efficacy most often report the response of the primary tumour, clinically however the primary tumour is removed, and tumour response is commonly reported as response rate or progression-free-survival, which is a measure of recurrent tumour progression.¹⁷³ The resulting patient overall survival and cause of death is therefore attributed to the recurrent tumour, which accumulating evidence suggests is biologically dissimilar to the primary tumour.²²⁰ It is therefore expected that treatments that are effective against the primary tumour may not show the same efficacy in the recurrent setting explaining the poor translation to clinical trials. The advancement of new therapies for glioblastoma therefore requires a deeper knowledge of the molecular mechanisms and novel targets of tumour recurrence. Advancements in this area, however, have been limited in humans due to the difficulty in obtaining quality tissue specimens and an inability to sensitively track clinical progression and identify the origin of tumour recurrence.²²¹ Animal models are necessary to overcome these limitations and further develop our understanding of recurrence biology. Currently, however, there are no standard mouse models available to study tumour recurrence with most models focussing on the primary tumour.

1.6 Identifying the origin of tumour recurrence

1.6.1 The role of glioblastoma infiltration

Since its early identification, glioblastoma has been known as a highly infiltrative disease. Early radical surgical interventions attempted to remove the entire inflected brain hemisphere only to witness recurrence from tumour cells that had crossed into the other hemisphere, providing evidence of extensive tumour infiltration far beyond the margins of the primary tumour.²²² In 1940, Scherer provided the most comprehensive description of tumour infiltration through observations from hundreds of clinical specimens.²²³ In his published work, Scherer described 4 pathways of glioblastoma invasion: through the normal parenchyma where they would collect just below the pial margin (subpial spread); through perineuronal and perivascular satellitosis, and through migration along white matter tracks

(intrafascicular spread). The patterns of glioma cell infiltration have since been referred to as the secondary structures of Scherer. The ultimate significance of this work demonstrated the capacity for individual glioblastoma cells to spread diffusely over long distances and into regions of brain whereby they could potentially form recurrent tumours. The formation of recurrent tumours is therefore recognised as the consequence of tumour infiltration and understanding the biology and mechanisms of tumour infiltration will be paramount to developing targeted therapies against tumour recurrence.

1.6.2 Identification of circulating tumour cells

Until recently hematogenous dissemination of glioblastoma cells was overlooked due to observation that glioblastoma does not typically metastasise, except in the rare gliosarcoma variant.²²⁴ Recent studies, however, have reported glioblastoma extraneural metastases in the lungs, lymph nodes, bone, liver and heart of glioblastoma patients.^{225,226} Furthermore, glioblastoma extraneural metastases have also been identified in the transplant recipients from glioblastoma donor patients, despite no evidence of glioblastoma tumour cells at the time of transplant.²²⁷ These cases highlight the tumourigenic potential of residual tumour cells from circulating tumour cell origin.

Recently, circulating tumour cells has also been described as an intrinsic feature of medulloblastoma cells.²²⁸ Garzia and colleagues further determined that these circulating tumour cell were capable of haematogenous dissemination and reseeding into the brain of a parabiosis xenograft system demonstrating a functional pathway for primary brain tumours to move in and out of circulation and infiltrate healthy areas of the brain. The findings of this study may prove to hold a similar biological mechanism in glioblastoma.

While the metastatic capacity of glioblastoma and the presence of circulating tumour cells are now widely reported in case studies, controversy still exists regarding the functional

relevance for glioblastoma development and progression, with no knowledge of the underlying mechanisms regulating this behaviour.²²⁹

1.6.3 Revisiting the tumour vasculature

Glioblastoma is considered to be one of the most vascularised solid malignancies, a property which is a distinguishing histological diagnostic criterion.³⁷ Angiogenesis was once thought to be the major contributor to glioblastoma neo-vascularisation and a promising therapeutic target with mechanisms well-studied in glioblastoma and other cancers.²³⁰ The ultimate failure of antiangiogenic inhibitors to treat glioblastoma, however, suggests that an incomplete understanding of the mechanisms and functional role of the tumour vasculature in glioblastoma.

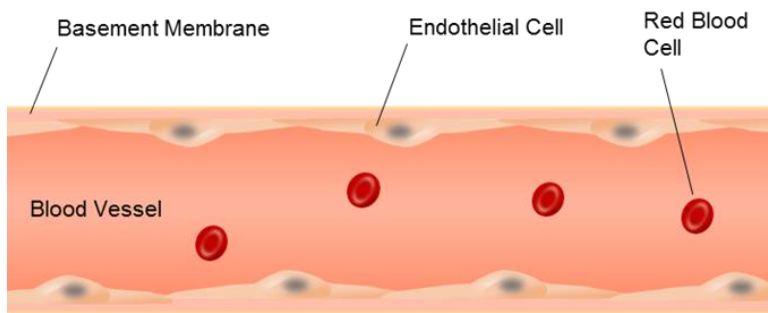
Microscopic examination of the tumour vasculature reveals morphologically dysfunctional vessels with extensive microvascular hyperplasia.^{228,231-233} Glomeruloid bodies are commonly observed, consisting of multiple layers of endothelial cells, pericytes and smooth muscle cells and displaying a thick basement membrane. Additionally, the blood vessels within the tumour are often dilated with loose endothelial cell junctions and/or the presence of fenestrations contributing significantly to parenchymal oedema.²³⁴ In contrast, normal vessels exhibit a monolayer of polarised and tightly interconnected adherent endothelial cells.²³⁵ Consequently, they deliver inadequate blood supply leading to hypoxia and acidification in some tumour regions.²³⁶ Tumour necrosis is often detected within the central tumour often concomitant with observations of haemorrhaging and thrombosed vessels.^{237,238} The necrotic process is considered a consequence of insufficient blood supply leading to reduced oxidative phosphorylation, an increase in inflammatory response factors and the loss of membrane integrity.²³⁹ The presence of necrosis has been shown to be a strong prognostic factor for glioblastoma survival and is also a key criteria for histological confirmation.^{41,240-242}

There is significant intratumoral heterogeneity in vascular morphology, and gliomeruloid bodies often coexist with more normal-appearing thin-walled tumour vessels.²⁴³ The blood–brain barrier, which is made up of endothelial cells, pericytes and astrocyte end-feet in normal brain vessels, is disrupted in glioblastoma, and vascular permeability is significantly increased.²⁴⁴ In the invasive front of the tumour and in the normal cortex that is frequently invaded by highly migratory glioblastoma cells, the blood–brain barrier is intact but extensive remodelling of the vasculature is present at recurrent formation.^{239,245,246}

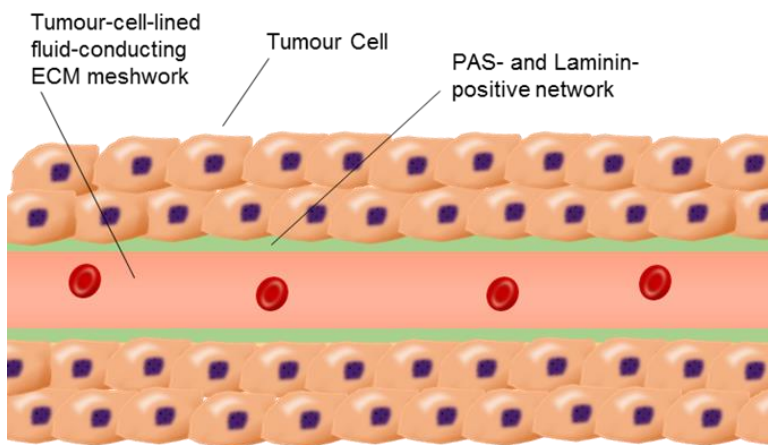
Intriguingly, along with these aberrant vasculature structures is the presence of tumour cells interconnected within the endothelium lining, termed mosaic blood vessels (Figure 1.1).^{247,248} Originally these tumour cells were thought to be due to invasion of the endothelium or exposure by fenestrations due to diminished structural integrity of tumour blood vessels.²⁴⁹ Recently it has been shown that these tumour cells express endothelial markers and in fact are capable of forming vascular structures without the need for endothelial cells, a process termed vasculogenic mimicry (Figure 1.1.).^{250,251}

The recent use of antiangiogenic inhibitors has produced only modest radiologic responses at best that have not translated to survival improvements.²⁵² These antiangiogenic inhibitors, however, are based on the pathology of tumour endothelial cells.²⁵²⁻²⁵⁴ It is plausible that tumour cell-lined blood vessels may not be regulated by the same pathways, making them resistant to antiangiogenic therapy. Indeed, some studies have demonstrated that these structures are non-responsive to Bevacizumab anti-VEGF treatment.^{255,256} Tumours may thus compensate for antiangiogenic inhibitors by utilising their own intrinsic ability to form blood vessels. Rather intriguingly the use of antiangiogenic inhibitors has also been found to enhance tumour invasion and metastasis.²⁵⁷ Exposure of tumour cells to circulation through tumour cell-lined blood vessels presents a novel opportunity for tumour dissemination and may suggest that the processes are intrinsically linked. To further understand the implications

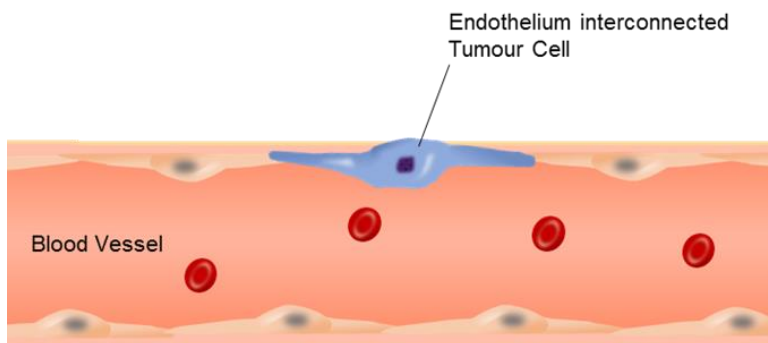
of vasculogenic mimicry we must determine the underlying mechanisms driving its formation.



Endothelial cell-lined Vasculature



Vasculogenic Mimicry



Mosaic Blood Vessel

Figure 1.1. Tumour cell associated blood vessel formations.

1.7 Vasculogenic mimicry

1.7.1 Identification and characterisation

Vasculogenic mimicry was first identified by Hendrix, Folberg and Maniotis through morphological, clinical and molecular characterisation of human uveal melanoma.²⁵¹ This comprehensive study demonstrated the ability of aggressive human melanoma cells to line patterned vascular channels of primary and metastatic tumours in absence of endothelial cells. Aggressive melanoma tumour cells but not poorly aggressive were observed to generate perfusable networks *in vitro*. Further molecular characterisation of these tumour cells revealed upregulation of endothelial cell-associated genes commonly expressed during embryonic vasculogenesis.²⁵⁸ Hendrix and colleagues therefore suggested that aggressive tumour cells capable of generating patterned vascular channels require a high degree of cellular plasticity.²⁵⁰ The authors also proposed that formation of these channels may provide a novel mode of microcirculation and pathway to metastasis.²⁵⁰

Since the initial conceptualisation of vasculogenic mimicry many studies have reported the existence of these histological structures in other types of aggressive cancer such as colorectal cancer, head and neck squamous cell carcinoma, breast cancer, ovarian cancer, ewing sarcoma and more recently gastric adenocarcinoma, Renal cell carcinoma, gallbladder carcinoma, merkel cell carcinoma, pancreatic cancer and orbital rhabdomyosarcoma.²⁵⁹⁻²⁷⁰ Though the occurrence of vasculogenic mimicry is rare within these tumours the presence of these structures has been shown to correlate with increased risk of metastasis and poorer prognosis.^{260,271-277}

1.7.2 Functional Relevance in solid tumours

The association of vasculogenic mimicry with poorer patient prognosis and more aggressive tumours suggests a functional advantage of vasculogenic mimicry in tumour progression. Indeed, the concept of vasculogenic mimicry relies on the ability of these structures to

provide angiogenesis-independent neovascularisation to the tumour. The idea of vasculogenic mimicry patterns as a pathway of perfusion in human tumours was initially met with controversy, though many studies have now reported functioning microcirculation independent of endothelial lined vessels in both mice and human tumours.^{270,278,279} Even with demonstration of functional microcirculation within these patterns some studies have questioned the clinical impact of vasculogenic mimicry given the rarity in which these structures are observed even within vasculogenic mimicry positive tumours.²⁴⁹ One interesting theory by Chen and colleagues suggests that vasculogenic mimicry represents early stages of rapid cancer growth.²⁸⁰ Chen et al., observed 3 types of microcirculation patterns in an intraocular melanoma mice model, consisting of either vasculogenic mimicry channels, mixed vasculogenic mimicry-endothelial cell-lined channels and endothelial cell-lined channels. Initial tumour growth was demarcated by vasculogenic mimicry formation only, which were replaced over time with endothelial cells transitioning from mosaic tumour blood vessels channels to complete endothelial cell lined blood vessels. Tumour growth was reported during each microcirculation pattern and necrosis was not evident, suggesting adequate blood supply during tumour progression. Notably these findings are consistent with mice embryonic vasculogenesis that demonstrated the presence of embryo vasculogenic mimicry during gestational development which was determined to be the main pattern of blood supply to the embryo during E5.5-E7.5. As the embryo expands endothelial cells proliferate and differentiate replacing embryo vasculogenic mimicry with mature endothelial cells. This process has been shown to be time-dependent and positively correlates with HIF1 α expression.²⁸¹ This study suggests that vasculogenic mimicry is not a novel process of neovascularisation but a host function that similarly to angiogenesis is hijacked for its favourable tumourigenic advantages. Interestingly this form of vasculogenesis was also reported in humans in 1998 through the observation that cytotrophoblasts underwent “pseudo-vasculogenesis”, whereby these cells transformed into endothelial-like cells and invade the uterus to access the maternal blood supply.²⁸² Taken together this suggests that

vasculogenesis is not substantiated by individual processes but rather a complex regulation of multiple pathways and cells to achieve the tight balance of homeostasis. In light of this novel mechanism of “vasculogenesis” it has been suggested that vasculogenic mimicry is the outcome of tumour cell phenotypic switching, through transdifferentiation into an endothelial-like phenotype.²⁶¹ Preventing the transdifferentiation of tumour cells to an endothelial-like phenotype represents a novel therapeutic strategy to both inhibit vasculogenic mimicry structures and associated tumour dissemination.

1.7.3 Vasculogenic mimicry and tumour cell plasticity

Transdifferentiation of tumour cells to vasculogenic mimicry phenotypes indicates a remarkable degree of tumour plasticity usually associated with embryonic stem cells.^{283,284} Tumours cells upregulate genes associated with embryonic progenitors while downregulating lineage-specific markers.²⁸⁵ Importantly they upregulate genes associated with angiogenesis and endothelial cell markers. Vasculogenic mimicry patterns are often also associated with stem-like markers CD133 and Nestin.^{261,286} Whether these markers indicate the involvement of cancer stem cells (CSCs) in vasculogenic mimicry formation is unclear, though the link between metastatic and highly aggressive tumours with formation of vasculogenic mimicry is well studied.^{287,288}

Cellular plasticity is suggested to be a limiting step for formation of vasculogenic mimicry and tumour cells are thought to first undergo transdifferentiation to an endothelial-like phenotype before they are capable of forming vasculogenic mimicry structures.^{261,286} We have termed this tumour cell transdifferentiation process “tumour endothelialisation”, to recapitulate the tumour cell plasticity to phenotypical and morphologically resemble endothelial cells. Notch and Nodal signaling pathways have been suggested as potential mechanisms for tumour endothelialisation due to their critical role in embryonic stem cell regulation.²⁸⁹ Nodal is generally absent from adult tissues but is found upregulated in highly aggressive cancers.^{290,291} Nodal is a secreted cytokine and part of TGF β family.²⁹² Activation

of Nodal signaling through ALK4/5/7 receptors induces phosphorylation of Smad2/3 and has been shown to promote EMT transdifferentiation and tumour aggression. Inhibition of ALK induced smad2/3 signalling reduces the ability of aggressive cancer cells to induce vasculogenic mimicry activity.²⁹³

The Notch signaling pathway is also involved in stem cell differentiation and self-renewal in embryonic tissue.²⁹⁴ During embryonic development Notch4 signaling upregulates Nodal expression to regulate tumour plasticity and aggression.²⁹⁵ Inhibition of Notch4 signalling *in vitro* abrogated vasculogenic mimicry formation, which could be rescued through overexpression of Nodal protein.²⁹⁵ Though these findings do implicate embryonic genes in vasculogenic mimicry they have yet to fully recapitulate the endothelialised phenotype and demonstrate the exact molecular signaling involved.

1.7.4 Vasculogenic mimicry and tumour metastasis

Increasing evidence suggests that vasculogenic mimicry is linked to initial cancer progression of tumour metastasis.²⁹⁶⁻³⁰⁰ Vasculogenic tumour cells are located within the exposed luminal of the vascular wall, whereby tumour dissemination into circulation is relatively simple.³⁰¹

The fact that vasculogenic mimicry is often formed by metastatic and highly aggressive tumour cells over poorly aggressive ones speculates that vasculogenic mimicry and metastasis may be inherently linked.³⁰² Epithelial-mesenchymal transition (EMT) is a process involved in metastasis and tumour progression. Recent reports suggest an inherent link between vasculogenic mimicry and EMT.²⁸⁷ Additionally the increase in metastasis observed from antiangiogenic inhibitors has been speculated to be caused by increased vasculogenic mimicry activity to compensate for tumour endothelial disruption.³⁰³ Prior short term treatment of sunitinib, a multi- tyrosine kinase inhibitor with antiangiogenic properties, with human breast or melanoma cancer cells were found to precondition these cells to a highly metastatic phenotype when implanted orthotopically into mice. Further treatment *in vivo* with sunitinib did not demonstrate any antiangiogenic activity. This is in stark contrast to the anti-

tumour and antiangiogenic effects observed with the same cancer cells not preconditioned with sunitinib treatment before implantation.³⁰⁴ Further evidence suggests that EMT regulators, such as TWIST, SNAIL and TGF- β are highly upregulated in vasculogenic mimicry forming tumours.³⁰⁵⁻³¹⁰ These genes are associated with stem-like features leading to loss of cell-cell contact and increased invasion.³¹¹ The expression of EMT inducer genes is also closely associated with tumour cell plasticity and vasculogenic mimicry formation.³¹²⁻³¹⁴ Though the association between vasculogenic mimicry and EMT/metastasis has been well correlated, studies have not identified the molecular pathways that link these two processes.³¹⁵ Due to the strong correlation between vasculogenic mimicry structures and EMT it is suggested that a common mechanism can both regulate tumour cell endothelialisation and EMT, two defined pathways of tumour plasticity.²⁸⁷ At minimum targeting both endothelialisation and EMT pathways would prove beneficial as a multi-strategy approach.

1.7.5 Signalling pathways in vasculogenic mimicry

Most of the molecular characterisation of vasculogenic cells began with the endothelial-specific receptors VE-cadherin and Eph2A.³¹⁶ Downregulation of either receptor inhibited vasculogenic mimicry formation.³¹⁷ Activation of VE-cadherin or Eph2A receptor phosphorylate PI3K/AKT and FAK signaling cascades converging on activation of Erk1/2 and increased MMP activity. MMPs cleave Laminin 5 γ 2 into γ 2' and γ 2x pro-migratory proteins.³¹⁸ MMP2, MMP14, Eph2A and laminin 5 γ 2 have been associated with short survival and more invasion cancer.^{276,319} Paradoxically MMP13 is also upregulated and cleaves laminin-5 enhancing tumour invasion while degrading VE-cadherin, which inhibits vasculogenic mimicry formation, suggesting negative regulatory pathways may be conserved. It is unknown if VE-cadherin or Eph2A are viable therapeutic targets due to the importance for structural integrity in quiescent endothelial cells.²³⁵ It is more likely that dysfunctional targets up- or down-stream would provide more options for therapeutic intervention. uPAR,

Bcl-2 and Twist 1 have also been shown to enhance VE-cadherin and vasculogenic mimicry expression while promoting EMT and correlating with poor prognosis in hepatocellular carcinomas and head, neck squamous cell carcinoma and large-cell lung cancer^{305,307,309,320}. The role of VEGF-A in vasculogenic mimicry is still controversial, while well characterized as a major driving force of angiogenesis, dysregulated tumour cells often express multiple VEGFR receptors that often have conflicting outcomes.³²¹ In melanoma carcinoma VEGF-A induced vasculogenic mimicry through noncanonical PI3K/PKC α signaling. Whereas in ovarian carcinoma VEGF-A promoted upregulation of endothelial cell specific genes, such as VE-cadherin, EphA2 and MMPs.³²² The overall importance of VEGF-A in vasculogenic mimicry formation is questioned by studies demonstrating a lack of response to the anti-VEGF inhibitor Bevacizumab, suggesting compensatory pathways are upregulated or that VEGF-A enhances the phenotype but is not a major driving force.^{256,323}

Wnt3a has also been demonstrated to induce vasculogenic mimicry in colon cancer through the wnt/ β -catenin signaling pathway by upregulating VEGFR2 and VE-cadherin.²⁹⁸ It has also previously been shown that wnt3a expression is associated with EMT providing a potential driving force for both vasculogenic mimicry and tumour dissemination.³²⁴ This association was identified in triple negative breast cancer whereby upregulation of wnt/ β -catenin pathway increased tumour metastasis through vasculogenic mimicry.³²⁵

Hypoxia is a key regulator of vasculature stabilization in both angiogenesis and vasculogenic mimicry.^{326,327} Under low oxygen conditions HIF1A or HIF2A translocalise to the nucleus and bind to hypoxia response elements, activating transcription of hypoxia target genes such as *VEGF-A*, *VEGFR1*, *EPHA2*, *TWIST* and *NODAL*.³²⁸ Hypoxic conditions have been shown to endothelialise cells through upregulation of EMT and stem-like markers, which induce formation of vasculogenic mimicry in a number of cancers.³²⁹⁻³³¹ Although hypoxia is considered one of the most potent stimuli to induce vasculogenic mimicry the mechanisms by

which cells become endothelialised are unclear.²⁸⁸ Recently it has been shown that Notch4 is activated by hypoxia feeding the Notch4-Nodal vasculogenic mimicry formation pathway.³³²

1.7.6 Therapeutic targeting of vasculogenic mimicry

Traditional antiangiogenic inhibitors such as endostatin, angiostatin and Combretastatin A4 phosphate, which have therapeutic efficacy for endothelial cells, have been shown to have little effect on vasculogenic mimicry patterns.^{319,333} Recently studies have targeted some of the known signaling pathways associated with the presence of vasculogenic mimicry. Norcantharidin was found to inhibit human gallbladder mice xenografts via suggested blocking of EphA2-FAK-Paxillin pathway and enhancement of TIMP2 activity.^{334,335} It has also been reported to antagonise tubular EMT by inhibiting TGF- β -induced smad2/3 signalling.³⁰⁶ Isoxanthohumol, an inhibitor of TGF- β signaling was also found to block vasculogenic mimicry formation in breast cancer cells.³³⁶ Cilengitide, the αv integrin inhibitor, was found to downregulate invasiveness and vasculogenic mimicry potential of melanoma cells through inhibition of $\alpha v \beta 5$ integrin.³³⁷ Gefinitib and LRIG1 have been shown to inhibit EGFR mediated vasculogenic mimicry formation *in vitro* through heat shock protein 27 and AKT/PI3K pathways respectively. Thalidomide has also been shown to inhibit vasculogenic mimicry in melanoma cells through inhibition of VEGF, MMP2 and MMP9 expression.³³⁸ The use of Genistein targeting VE-cadherin expression has also been suggested to inhibit vasculogenic mimicry.³³⁹ Interestingly it has been reported that sunitinib, a multiple tyrosine receptor kinase inhibitor against VEGFR, PDGFR, FGFR has been able to prevent vasculogenic mimicry formation of triple negative breast carcinoma cells, which are resistant to bevacizumab treatment.³⁴⁰ Though this suggests a potential role for PDGFR and FGFR, vasculogenic mimicry resistance to sunitinib has been observed in renal carcinoma and subsequent everolimus second-line therapy was shown to reduce vasculogenic mimicry activity.³⁴¹ The inconsistency of therapeutic efficacy is one problem associated with our lack of understanding of the driving mechanisms for vasculogenic mimicry. By identifying the

plasticity pathways initiating tumour endothelialisation we can prevent the formation of vasculogenic mimicry and provide targeted therapeutic strategies.

1.7.7 Vasculogenic Mimicry in Glioblastoma

Traditionally newly formed vessels in glioblastoma were thought to arise from pre-existing blood vessels.³⁴² Recently vasculogenic mimicry has been identified in glioblastoma patients with a reported incidence of 65.9%.^{343,344} CD133+ stem-like glioblastoma cells isolated from tissue samples demonstrated a high degree of plasticity transdifferentiating into endothelialised cells and vascular smooth muscle-like cells. Vasculogenic mimicry channels have also been found to highly express VEGFR2, which is important for glioma cells to maintain “stemness”.^{345,346} Common vasculogenic mimicry markers VEGFR2, PDGFR, EGFR but not CD31 were observed in glioblastoma samples recapitulate those found in other cancers expressing.³⁴⁷ There is already evidence to suggest that the presence of vasculogenic mimicry confers to poorer overall survival in glioblastoma, though the mechanisms contributing to this process are not understood.^{348,349} Most recently at the ASNO-COGNO scientific meeting 2016, Chen et al., reported that Glioblastoma patients exhibiting vasculogenic mimicry had a median survival 4 months less than those without.³⁵⁰ This study demonstrates the association of vasculogenic mimicry with more aggressive tumours but also highlights a potential novel therapeutic strategy for glioblastoma.

There has been particular focus on glioma stem cells (GSCs) as a source of vasculogenic mimicry due to the association with CD133 expression.³⁵¹ One speculated mechanism for transdifferentiation has been through notch signaling, due to its involvement in GSC maintenance. Wang et al., demonstrated that blocking notch-1 signaling with γ -secretase inhibitors, prevented early stages of GSC transdifferentiation to endothelialised progenitor cells.²⁸⁶ Complicating things further bevacizumab treatment blocked differentiation of endothelialised progenitor cells to mature endothelialised cells. These results suggest a two stage process for GSC transition into an endothelialised phenotype capable of forming

vasculogenic mimicry. Furthermore endothelialised cells were more frequently detected in hypoxic regions of the tumour and associated with the stem-like marker CD144.³⁵² This process was observed to be independent of VEGF- or FGF-mediated signaling. Along with the expression of GSC markers CD133 and CD144 hypoxic regions around the invading pseudopalisading necrotic area have been shown to highly express TGF- β and EMT-related transcriptional factor TWIST.³⁵³

Recently it has been shown that TGF- β signaling can regulate vasculogenic mimicry in GBM. TGF- β has been found to induce an endothelialised phenotype in U251 cells and inhibition through Galunisertib abrogated vasculogenic mimicry activity *in vitro* and tumour size *in vivo*.^{256,354} Despite having known angiogenic effects and being a key regulator of EMT, the role of TGF- β in vasculogenic mimicry formation has been speculated but not fully explored.^{336,354,355} Further understanding of the influence of TGF- β on vasculogenic mimicry may provide insight into its driving mechanisms.

1.7.8 Canonical TGF- β signalling in endothelial cells

TGF- β signaling plays a role in angiogenesis and microvascular modulation. In endothelial cells TGF β signal through two type I receptors, Activin-like receptor- (ALK) 1 and 5 and their accessory receptor endoglin, which enhances ALK receptor activity.³⁵⁶⁻³⁵⁹ *In vivo* mice studies demonstrate that loss of components of TGF- β signaling result in gestational death due abnormal vascular development presenting with dilated and leaky vascular.³⁵⁷ Similarly, loss of function or deletion of TGF- β type I receptor ALK1 and its accessory receptor endoglin are associated with *hereditary hemorrhagic telangiectasia* (HTT) in humans, a systemic disease characterised by arteriovenous malformations and telangiectases in organs.³⁶⁰ This vascular condition has been successfully modelled in mice through specific endothelial cell deletion of ALK1 and endoglin genes.^{356,361} Both ALK1 and ALK5 are postulated to be vital for activation and survival of endothelial cells (Figure 1.2).³⁶² ALK5 is ubiquitous in human cells while ALK1 has been observed to be restricted to endothelial cells

and sites of embryonic angiogenesis.³⁶³ TGF- β /ALK5 induces smad2/3 phosphorylation, which blocks angiogenesis and endothelial cell proliferation, leading to a resolute quiescent state as seen in mature blood vessels.^{362,364,365} VE-cadherin has also been found to activate TGF- β -Smad2/3 in endothelial cells, which is speculated to promote endothelium stabilization.³⁶⁶ In contrast to TGF- β /ALK5, TGF- β /ALK1 signalling induces smad1/5 activation stimulating proliferation, migration and angiogenesis.³⁶⁷ Although these two signaling pathways have opposing effects a fine balance is needed for endothelial cell function and disruption of either pathway can lead to TGF- β impaired responses.³⁶² Furthermore, endoglin is required for normal function in endothelial cells and suppression of endoglin impairs TGF- β /ALK1 responses and provides inhibition of the TGF- β /ALK5 pathway.^{368,369} The role of TGF- β has been well documented in angiogenesis and blood vessel stabilization, with particular respect to the ALK1 and ALK5 signalling pathways.

1.7.9 TGF- β signalling in glioblastoma tumour cells

The TGF- β signalling pathway is aberrantly active in glioblastoma tumours. Both TGF- β and type I and II TGF- β receptors (T β RI and T β RII, respectively) are found highly expressed in glioblastoma tissue compared to non-tumour tissue.^{370,371} Immunohistochemistry staining, shows enhanced expression of TGF- β ligand and receptors in spindle-shaped tumour cells, surrounding proliferating vessels or around areas of necrosis, in addition to cells clustering around the advancing tumour margin.^{353,370}

TGF- β stimulation have been shown to mediate glioblastoma migration and invasion both in *in vitro* and *in vivo* models.^{372,373} Knockdown of *TGF β RII* was shown to inhibit TGF- β -induced glioblastoma invasion and migration *in vitro* and in T-98G and C6 glioblastoma animal models.³⁷³ Interestingly, stable knockdown of *TGF β RII* also reduced tumourigenicity of rat C6 cells by 50% in mouse models. In line with this finding, Peñuelas and colleagues also reported TGF- β induced self-renewal of glioblastoma-initiating cells through Smad-mediated upregulation of leukemia inhibitory factor (LIF).³⁷⁴ Furthermore, in glioblastoma

CD44⁺ neurospheres, TGF- β has been found to induce transcription of *ID1* through a TGF- β -responsive element located at the same promoter region described to suppress *ID1* transcription in epithelial cells.^{375,376} CD44⁺/ID1⁺ neurospheres were found to be located in perivascular niches *in vivo* and inhibition of the TGF- β pathway decreased *ID1*, *CD44* and *ID3* levels in tumours while preventing tumour recurrence.³⁷⁶ High Id1 and CD44 levels correlated with poorer prognosis in glioblastoma patients. In another study by Ikushima *et al*, TGF- β was found to regulate stemness by inducing Sox4/Sox2 expression.³⁷⁷ In mice studies intracranial inoculation of glioblastoma-initiating cells pretreated with SB431542, a T β RI inhibitor, showed no evidence of tumour burden by pathological examination with H&E staining, nor presented with any neurological symptoms.

TGF β 1 expression in glioblastoma tumours is also a key signature gene associated with the mesenchymal subtype.³² In a study by Joseph *et al.*, TGF- β was found to mediate transdifferentiation from a proneural phenotype to mesenchymal through ZEB1.³⁷⁸ The mediated shift to the mesenchymal phenotype was associated with enhanced migration and invasion in intracranial mouse models. Additionally, pSmad2, ZEB1 and the stemness marker YKL40 were found to have overlapping expression in patients samples, localized around perivascular niches. Histopathological examination of TGF- β treated cells inoculated intracranially into mice, also displayed denser vasculature compared to untreated or combined A8301, a small inhibitor of T β RI, and TGF- β treatment.

There is conflicting information regarding the prognostic relevance of measuring TGF- β activity in glioblastoma. Roy *et al.*, reported a statistical significant survival differences of 11.9 months in high and intermediate *TGF β 1* expressing tumours and 23 months in low *TGF β 1* expressing tumours.³⁷¹ This finding, however, was inconsistent with another study by Frei *et al.*, who did not find any survival correlation when comparing high and low levels of TGF- β mRNA and protein expression.³⁷⁹ Roy and colleagues speculated that the different findings may be caused by the cutoff criteria used to separate high and low TGF- β expression

levels.³⁷¹ In a study of 25 glioma tumours Bruna *et al.*, reported a positive correlation with pSmad2 levels and glioma grade, with high pSmad2 levels conferring poorer prognosis.³⁸⁰

While TGF- β signalling has been associated with aggressive tumour phenotypes, there is conflicting evidence regarding its prognostic capabilities. Recent advancements in our understanding of glioblastoma plasticity and cellular switching point towards the increased relevance of temporal and spatial regulation of gene expression, which cannot be captured in static sampling.

In contrast to the well-established TGF- β -Smad2/3 signalling pathway in glioblastoma, recent studies have identified expanded TGF- β -Smad1/5 signalling in some glioblastoma cells.³⁸¹

The TGF- β -Smad1/5 signalling pathway, which is normally restricted to endothelial and embryological cells, is transcriptionally functional and is concomitantly activated with Smad2/3 signalling. The biological relevance of this expanded signalling pathway is not yet known.

1.8 Summary

In summary, patient care for glioblastoma remains dismally poor with the Stupp protocol remaining the gold standard in clinical care for the last 15 years. Despite the almost ubiquitous use of the Stupp protocol for glioblastoma treatment, there is inconsistency in the reported overall survival outcomes for patients receiving the Stupp protocol raising questions regarding its overall efficacy in the general population. It is also concerning that in the development of new treatments current clinical trials report overwhelming negative results. This is despite positive preclinical studies, suggesting that preclinical models may not accurately recapitulate glioblastoma biology. Additionally, the failure of antiangiogenic inhibitors suggests that the pathological relevance of glioblastoma vascularity may be related to an alternate mechanism that requires further exploration. An improved understanding of current therapies and glioblastoma biology will provide a strong foundation for the discovery of key glioblastoma progression mechanisms and improved translation into therapeutic care.

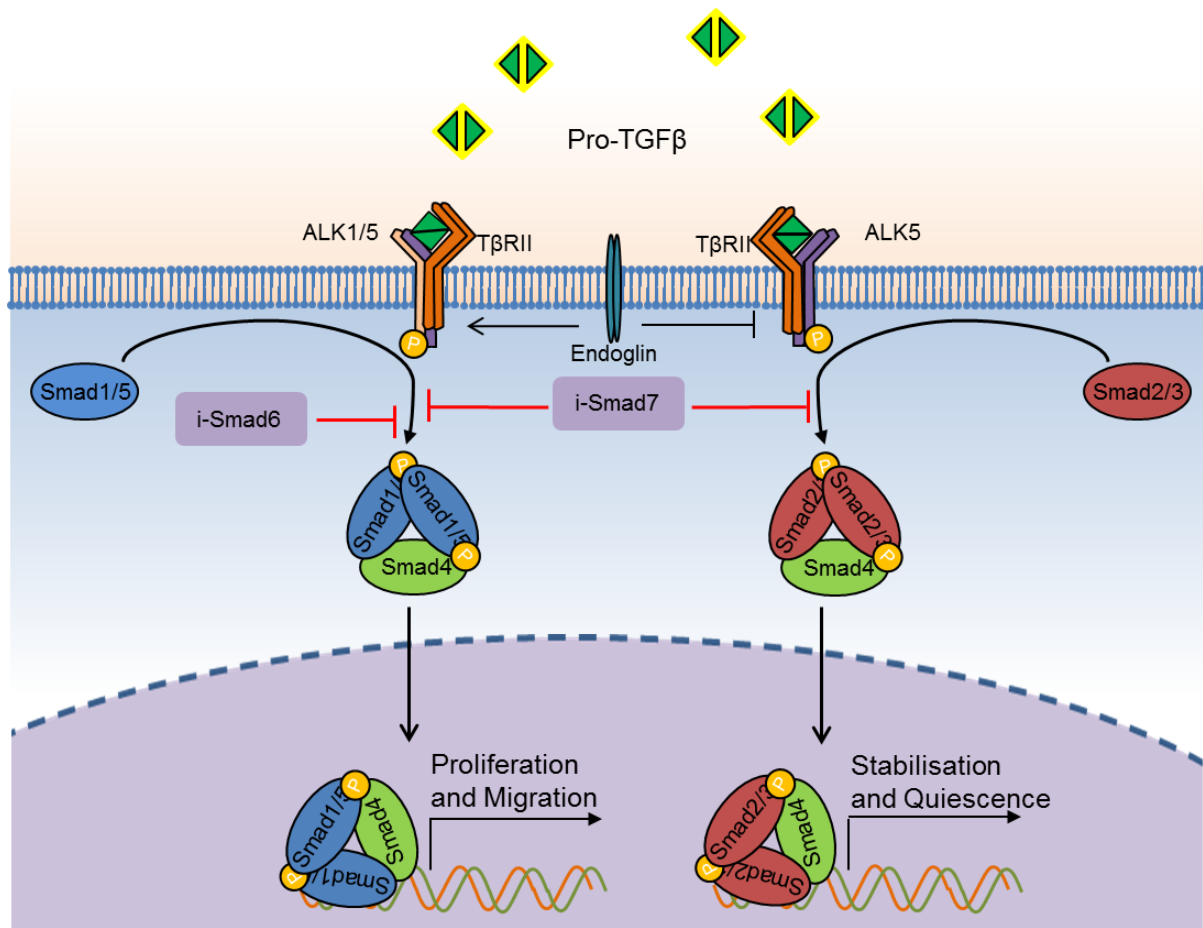


Figure 1.2. Canonical TGF- β signalling pathways in endothelial cells

Hypothesis and Aims

This thesis addresses the following hypothesis:

Chapter 3: Glioblastoma treatment: a road to nowhere

Hypothesis: Overall survival for glioblastoma is stagnating and the current standard of care has not provided a significant benefit for long term survivors

Chapter 4: A new systemic disease mouse model for glioblastoma capable of single tumour cell detection

Hypothesis: Glioblastoma is a systemic brain disease, consisting of single cell infiltration to all regions of the supratentorial brain.

Chapter 5: TGF- β -ALK1-Smad1/5 signalling regulates glioblastoma endothelialisation and systemic brain dissemination

Hypothesis: Activation of TGF- β -smad1/5 signalling regulates glioblastoma endothelialisation and infiltration into peripheral brain regions

The specific aims of the study are:

1. To determine if glioblastoma overall survival has improved since the introduction of the Stupp protocol
2. To develop a mouse model to investigate glioblastoma infiltration biology and molecular mechanisms
3. To evaluate the role of TGF- β -smad1/5 signalling in glioblastoma endothelialisation and investigate its function in whole brain tumour infiltration

Chapter 2: Materials and Methods

2.1 Clinical trial literature search

We performed a literature search using Pubmed with the MeSH terms: *glioblastoma* OR *gbm* AND *clinical trial* AND *stupp* OR *temozolomide*. Abstracts were reviewed for all titles referring to an original clinical trial study. Data was extracted for clinical trials using the Stupp protocol as the experimental or comparative arm where median overall survival with 95% confidence intervals were reported. Clinical trials restricted to glioblastoma in the elderly were excluded from analysis.

2.2 Clinical data

Patient data was obtained from the 18 SEER registry database and for incidence rates the NPCR data base was also utilised (November 2018 submission). Selected glioblastoma patients were histologically confirmed and classified as ICD-0-3 9440-9442 with malignant behaviour (/3), diagnostic age 20 years and above and diagnosed between the January 2000 to December 2016 (or January 2001 to December 2016 for incidence data). Patients that only had autopsy or death certificates or glioblastoma was not recorded as the first and only primary tumour were excluded from analysis.

Clinical glioblastoma data was also queried from The Cancer Genome Atlas (TCGA) database using the R package *TCGAbiolinks*. Patient data was collected for glioblastoma cases (ICD-0-3 9440/3) with known survival times and complete treatment history.

2.3 Cells and cell culture

Human glioblastoma cell lines MU20 and MU41 were derived from patients at the Royal Melbourne Hospital (Parkville) and U87MG obtained from the ATCC (American Type Culture Collection). All cells were maintained in Dulbecco's modified Eagle's medium

(DMEM, 11965092, ThermoFisher Scientific) and contained 10% fetal bovine serum (FBS, 16000044, ThermoFisher Scientific) (DKSH), 100U/ml penicillin and 100µl/ml streptomycin (Pen/Strep, 15140148, ThermoFisher Scientific). Cells were incubated in a humidified atmosphere of 10% CO₂ 37°C.

2.4 Antibodies and reagents

Goat polyclonal antibodies anti-smad6, anti-smad7 and anti-smad5 were purchased from Santa Cruz Biotechnology (sc6034, 1:1000; sc7004, 1:1000; sc7443, 1:1000). Mouse monoclonal anti-smad2 antibody was purchased from Transduction Laboratories (S66220, 1:1000). Rabbit polyclonal anti-phospho-smad2/3 (PS2) and anti-phospho-smad1/5 (PS1) antibodies were gifted from Prof. Peter Ten Dijke (Leiden University Medical Center, Netherlands). Monoclonal mouse anti-actin antibody was purchased from Sigma-Aldrich (A-4700, 1:1000). HRP-conjugated secondary antibodies (1:5000) were purchased from Bio-Rad.

Human recombinant TGFβ1 and human recombinant BMP7 was purchased from PeproTech (100-21-100; 120-03-10). Adenovirus vectors Ad-CMV-Tomato, Ad-BRE-Tomato, Ad-BRE-Firefly-Luciferase, Ad-CAGA-Gaussia-Luciferase, Ad-CMV-Smad6 and Ad-CMV-Smad7 were all generated by the Zhu lab. FuGENE® HD transfection reagent was purchased from Promega (E2312).

2.5 Western blotting analysis

Cells were lysed in lysis buffer (30mM HEPES, 1% Triton-X-100, 2mM MgCl, 150mM NaCl, 5mM EDTA, complete protease inhibitor cocktail (Roche, Switzerland) on ice for 30mins. Lysates were centrifuged at 13000rpm for 15mins and supernatant extracted. Lysates were heated at 95°C for 10 minutes Laemmli sample buffer (Bio-Rad Laboratories, Australia). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Australia). After blocking with 4% skim milk for 1h at room

temperature, membranes were probed with primary and secondary antibodies. The signal was developed using Western Lightning® ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer, USA). All western blotting results were representative of three separate experiments.

2.6 Adenovirus fluorescence and immunofluorescence

Cells were transduced with Ad-tomato and Ad-eGFP containing virus in 5% FBS DMEM media. Optimal expression was provided after 48hr infection.

For *in vitro* immunofluorescence cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, USA) for 5-7mins and blocked in 1% BSA for 1hr at room temperature. Cells were probed with primary and ALEXA fluorescence secondary antibodies. Fluorescent signal was detected using Leica fluorescent microscope.

For tissue section immunofluorescence, fresh frozen tissue is sectioned at 6microns and fixed in 100% acetone at -20°C for 10mins. Sections are blocked in 5% normal serum and probed with primary and ALEXA secondary antibodies. Slides are stained with 1microg/microL Hoescht for 5mins. Sections are mounted using VectaShield Antifade Mounting Media for Fluorescence (Vector Laboratories, USA) and sealed using clear nail polish. Fluorescent signal was detected using Leica fluorescent microscope and Leica sp5 Confocal microscope.

2.7 Luciferase assay

Reporter cells (5000 cells/well in 96-well plate) were seeded and transduced with Ad-BRE-Firefly-Luciferase virus (MOI: 250) and Ad-pCAGA-Gaussia-Luciferase virus (MOI: 100) in 5% FBS DMEM media. After 24hr infection, cells were serum starved in 100µl serum free DMEM media for 16hrs. Reporter cells were stimulated with ligand (TGFβ1 or BMP7) for a further 6hrs. Thereafter, cells were lysed and assessed for luciferase activity using Luciferase Assay Kit (Promega, USA) following the manufacturer's instructions. Luciferase activity was presented as the fold change as compared with control.

2.8 *In vitro* lattice formation assay

48-well tissue culture plate were coated with Matrigel Basement Membrane Matrix (100µl/well, BD Bioscience, Australia) which was allowed to polymerise at 37°C for 1hr. Cells were resuspended and seeded on Matrigel at 4.5×10^4 cells/ml in 5% FCS DMEM media and incubated for 6hrs at 37°C. To investigate the effects of inhibitory smads on lattice formation, cells were transduced with Ad-CMV-TOM, Ad-Smad6 and Ad-Smad7 48 hrs prior to seeding. Cultures were photographed using a Leica fluorescence microscope. Images were quantified using angioogenesis analyzer <http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>.

2.9 Stable transfection of firefly and gaussia luciferase

U87MG cells were stably transfected with pGL4.51[luc2/CMV/Neo] (Promega, Australia) or pCMV-Gluc-KDEL (Prolume Ltd., Pinetop, AZ, USA) vector using Eugene® HD (Promega). Cells underwent selection with 2mg/ml G418 (Roche, Switzerland) and stable clonal colonies obtained.

2.10 Animal experimentation

Animal experimentation has been approved by the University of Melbourne Animal Ethics Committee (1614055). 6-8 week female NOD-SCID mice were purchased from Animal Resource Centre (ARC) Canning Vale, Western Australia and housed in the Department of Surgery Animal Facility, University of Melbourne, Victoria. For intracranial implantation, mice were anaesthetized with 4% isoflurane and maintained at 2% isoflurane for the procedure. Mice were shaved and positioned on the stereotactic. A small burr hole <1mm diameter was fashioned and 5×10^4 firefly-labelled cells implanted in 5µL using a 27 gauge needle at a flow rate of 1µL/min. The needle was retracted and the hole sealed using sterile bone wax. Mice were monitored for recovery and 5 days later a non-labelled contralateral tumour was implanted using the same procedure. Once recovered mice were

transferred to Monash Institute of Pharmaceutical Sciences for IVIS imaging and blood collection. 150mg/kg D-luciferin was i.p injected into mice for IVIS illumina II (Caliper Life Sciences, USA) bioluminescent detection.

Following experimental end-point, mice are euthanized and brains harvested. The firefly-labelled tumour is frozen in OCT and sectioned for immunofluorescence. Remaining tissue is snap-frozen for *ex vivo* analysis.

2.11 *Ex vivo* luciferase analysis of tissue samples

Frozen tissue was cut into ~25mg pieces and homogenised in cell culture lysis reagent (Promega, USA). Tissue lysates were placed at 4°C for 30mins with agitation and transferred to an opaque 96 well plate for luminescence reading using a luciferase assay kit (Promega) and luminometer (Promega). Luciferase activity is presented as relative luciferase units (R.L.U).

2.12 Human glioblastoma images

Human glioblastoma images of *in situ* hybridization and H&E tissue sections were obtained from the publicly available Ivy Glioblastoma Atlas Project (Ivy GAP) (<https://glioblastoma.alleninstitute.org/>). Tissue samples were analysed for the following gene expressions: *FGFR3-TACC3*, *MECOM*, *ENPEP*, *EPAS1*, *GFAP*.

2.13 Statistical analysis

All experiments were determined three times. The data were recorded as mean $\pm$ SEM and statistical analysis was performed using Graphpad Prism 6 or R software (<https://www.r-project.org/>). Differences between groups were analysed by one-way ANOVA with Tukey's multi-comparisons test or using an unpaired student's t test. Single cell quantitation was determined using best-fit linear regression analysis and cell number extrapolated for positive luciferase samples. P value < 0.05 was considered statistically significant.

For registry data analysis, age-adjusted incidence rates to standardised to the US 2010 census population using the following formula:

$$aarate_{x-y} = \sum_{i=1}^y \left(\frac{count_i}{pop_i} \right) \times 100,000 \times \left(\frac{std_{std_i}}{\sum_{j=1}^y std_{std_j}} \right)$$

Age-adjusted confidence intervals were calculated using the Fay and Feuer method (1997).³⁸² SEER's Joinpoint Regression Program (4.7.0.0) was used to determine annual percentage changes for incidence rates, hazards ratio and survival rates over time. Age-adjusted relative survival and conditional survival rates were calculated by SEER*Stat software (8.3.6) using the International Cancer Survival Standard (ICSS) 2 with 5 default age groups. Cox regression estimated hazard ratios and Kaplan-Meier analysis was performed in R using the *survival* and *survminer* packages. The relative hazard rate, cumulative log hazard risk and survival probability curves were performed using the R package *pamtools*. Graphs were generated using Graphpad Prism 6 and R Software. The null hypothesis was rejected when $P < 0.05$.

Chapter 3: Glioblastoma treatment: a road to nowhere

Key Points

Question Is glioblastoma treatment working?

Findings Analysis of a population-based registry data of 35,236 glioblastoma cases, demonstrated limited short-term survival benefit with the Stupp protocol that did not translate into improved survival beyond 2 years. Despite this finding, survival shown improvements independent of the Stupp protocol from 2000 – 2009 but this improvement trend has stagnated.

Conclusion Current therapies do not target the root-cause of tumour progression.

3.1 Abstract

Importance Glioblastoma, a WHO grade IV primary brain tumour, remains as one of the most aggressive forms of human cancer. Despite intensive research few advances have been made to therapeutic care since the introduction of the Stupp protocol in 2005. Even with this standard of care, clinical trials often report highly variable survival outcomes in contrast to the original EORTC-NCIC phase III clinical trial leading to discrepancies in the reported benefit.

Objective To analysis trends in glioblastoma survival and the impact of the Stupp protocol in real-world population-based registry data to establish the current status of glioblastoma treatments.

Design, Setting and Participants We obtained adult glioblastoma patient data from the 18 Surveillance, Epidemiology and End Results (SEER) registries diagnosed between the years 2000 – 2016 and for incidence data we also complemented the SEER registries with the National Program of Cancer Registries. All cases were microscopically confirmed

glioblastoma (ICD-O-3 codes 9440-9442, /3), had active follow-up and was recorded as the first and only primary tumour. The Cancer Genome Atlas was also queried for glioblastoma patients with survival data and complete treatment history.

Main Outcomes and Measures Incidence data by year of diagnosis was standardised to the US 2010 census population. Comparative analysis of patient survival was performed using cox regression hazards ratio, Kaplan-Meier analysis and age-standardised conditional and relative survival rates.

Results Through analysis of 35,236 glioblastoma patients diagnosed between the year 2000 – 2016, our results demonstrate an early and consistent improvement in short-term survival from 2000 – 2009, preceding the introduction of the Stupp protocol. This survival improvement trend is also observed when selecting only for patients that received the full standard of care. These survival improvements, however, stagnate in recent years with little to no advancements made from 2009 – 2016. Significantly, when analysing patients surviving 2 years or more from diagnosis (long-term survivors), representing ~20% of all cases, there is no survival improvement observed from 2000 – 2016. In cases diagnosed between 2010 – 2016, multivariate analysis revealed no associated benefit with radiotherapy or chemotherapy in long-term survivors. Only age and total resection remained significant prognostic factors in this cohort. Non-proportional hazards modelling also showed no long-term benefit with surgery, radiotherapy or chemotherapy, with only reduced risk of death conferred to the first 18 months post-diagnosis. These survival outcomes were consistent across different age-groups.

Conclusions and Relevance Together these results suggest that the Stupp protocol has provided limited benefit to patient survival and discouragingly show that there has been no improvement for long-term survivors. Current research strategies and therapeutic development are failing to provide needed improvements to glioblastoma care, and there is a still significantly gaps in our understanding of the major mechanisms driving tumour

progression. Future therapeutic will need to identify new biological targets to meaningful impact to glioblastoma survival.

3.2 Introduction

Glioblastoma is a WHO grade IV primary brain tumour and the most common glioma in adults.^{383,384} It is one of the most aggressive forms of human cancers, resulting in one of the poorest survival outcomes for any solid malignancy.³⁸⁴ The Stupp protocol remains the current standard of care consisting of surgery and radiotherapy with concomitant and adjuvant temozolomide, which was FDA approved for newly diagnosed glioblastoma in 2005.⁹¹ The EORTC-NCIC clinical trial reported a median overall survival benefit of 2.5 months with the addition of temozolomide compared to surgery and radiotherapy alone. Further follow-up of patients proceeding the initial publication found 2-year overall survival rates of 27.2% and 9.8% with temozolomide versus 10.9% and 1.9% without, which was hailed as a significant benefit to long-term survival.

Population-based analysis are readily utilised to examine the real-world response to new treatments. Previous registry studies have compared survival rates of pre-temozolomide and post-temozolomide periods, concluding that survival rates have increased over the period, though to a lesser extent than reported in the original EORTC-NCIC clinical trial. The 2- year survival rate for registry data have reported an increase from 13% to 17% following the introduction of the Stupp protocol. Intriguingly 5-year survival rates within large registry datasets have remained relatively unchanged with chemotherapy use in the Stupp era. These studies raise questions about the true effect of the Stupp protocol in the population and point to a stagnation in long-term survival rates. Confoundingly recent clinical trials using the Stupp protocol have also demonstrated increasing median overall survival trends over time, suggesting that factors independent of Stupp protocol usage may be have improved survival outcome for glioblastoma patients (Figure 3.1). Additionally, these clinical trials also report a large variation in reported median overall survival between and within clinical studies suggesting that there may be a large variability in response rates to the Stupp protocol.

The primary goal of this study was to investigate survival trends between 2000 to 2016 to determine the overall contribution of the Stupp protocol to improved survival outcomes for patients in addition to understanding overall glioblastoma survival trends over time. This study will also investigate survival trends in long-term survivors and identify prognostic factors associated with improved survival in this cohort.

3.3 Methods

Clinical Trial Literature Search

We performed a literature search using Pubmed with the MeSH terms: *glioblastoma* OR *gbm* AND *clinical trial* AND *stupp* OR *temozolomide*. Abstracts were reviewed for all titles referring to an original clinical trial study. Data was extracted for clinical trials using the Stupp protocol as the experimental or comparative arm where median overall survival with 95% confidence intervals were reported. Clinical trials restricted to glioblastoma in the elderly were excluded from analysis.

Patient Population Registry Datasets

Patient data was obtained from the 18 SEER registry database and for incidence rates the NPCR data base was also utilised (November 2018 submission). Selected glioblastoma patients were histologically confirmed and classified as ICD-0-3 9440-9442 with malignant behaviour (/3), diagnostic age 20 years and above and diagnosed between the January 2000 to December 2016 (or January 2001 to December 2016 for incidence data). Patients that only had autopsy or death certificates or glioblastoma was not recorded as the first and only primary tumour were excluded from analysis.

Clinical glioblastoma data was also queried from The Cancer Genome Atlas (TCGA) database using the R package *TCGAbiolinks*. Patient data was collected for glioblastoma cases (ICD-0-3 9440/3) with known survival times and complete treatment history.

Statistical Analysis

Age-Adjusted incidence rates to standardised to the US 2010 census population using the following formula:

$$aarate_{x-y} = \sum_{i=1}^p \left[\left(\frac{count_i}{pop_i} \right) \times 100,000 \times \left(\frac{std_{std_i}}{\sum_{j=1}^p std_{std_j}} \right) \right]$$

Age-adjusted confidence intervals were calculated using the Fay and Feuer method (1997).³⁸² SEER's Joinpoint Regression Program (4.7.0.0) was used to determine annual percentage changes for incidence rates, hazards ratio and survival rates over time. Age-adjusted relative survival and conditional survival rates were calculated by SEER*Stat software (8.3.6) using the International Cancer Survival Standard (ICSS) 2 with 5 default age groups. Cox regression estimated hazard ratios and Kaplan-Meier analysis was performed in R using the *survival* and *survminer* packages. The relative hazard rate, cumulative log hazard risk and survival probability curves were performed using the R package *pamtools*. Graphs were generated using Graphpad Prism 6 and R Software (<https://www.r-project.org/>). The null hypothesis was rejected when $P < 0.05$.

3.4 Results

Glioblastoma Incidence

Glioblastoma incidence counts were obtained from the NPCR and SEER database, which together form the US Cancer Statistics database. Age-adjusted incidence rates were standardised to the most recent US 2010 census population. Overall glioblastoma age-adjusted incidence rates (AAIR) had an annual percentage increase of 1.3% (95% CI, 0.4 to 2.3%; $P < 0.05$) in the years 2001 to 2005 (AAIR, 4.16 to 4.38, [95% CI, 4.07 to 4.26 & 4.29 to 4.47 respectively]), following which incidence rates remained stable (from 2005 to 2016) (Figure 3.2A). Male glioblastoma age-adjusted incidence rates increased slightly from 2001 to 2016 with an annual percentage increase of 0.2% (95% CI, 0.0 to 2.3%; $P < 0.05$) (AAIR, 5.05 to 5.27, [95% CI, 4.90 to 5.20 & 5.15 to 5.40 respectively]), while Female age-adjusted incidence rates increased from 2001 to 2006 with an annual percentage increase of 1.6% (95% CI, 0.3 to 2.9%; $P < 0.05$) (AAIR, 3.34 to 3.51, [95% CI, 3.22 to 3.47 & 3.51 – 3.74 respectively]), following which incidence rates stabilised between 2006 to 2016 (Figure 3.1B). Glioblastoma incidence rates also increased within specific age-groups (20-39, 60-69, 70-79 and 80+ years old) between 2001 and 2016 (Supplementary Table 3.1 & Supplementary Table 3.3). The overall Male to Female incidence ratio remained consistent from 2001 to 2016 (Supplementary Table 3.2).

Patient Selection

For survival analysis the final cohort was selected from 18 SEER registries selecting for glioblastoma cases diagnosed between the years 2000 to 2016 with ICD-0-3 coding 9440/3, 9441/3 and 9442/3 (Figure 3.3). Exclusion criteria included not first primary cancer, autopsy or death certificate only and not microscopically confirmed. 35,236 cases met the inclusion criteria and key patient characteristics are detailed in Supplementary Table 3.4.

Survival Analysis (cohort diagnosed in 2000 to 2016)

We utilised the age-adjusted hazard ratio to compare the overall risk of death for glioblastoma cases diagnosed between 2000 to 2016. The year 2000 was used as the reference comparison. From 2000 to 2016 there is an overall decrease trend in the hazard ratio, with a significant decrease observed between the years 2000 to 2005, prior to the approval of the Stupp protocol (Figure 3.4A). The hazards ratio dropped by -7.1% per year from 2001-2005 (95% CI, -8.8 to -5.4%; $P<0.05$) and decreased further by -1.9% from 2005 to 2016 (95% CI, -2.4 to -1.4%; $P<0.05$). 1- 2- and 5-year age-standardised relative survival rates (ASRSR) also increased prior to the introduction of the Stupp protocol (1-year ASRSR, 2000 to 2009, 4.2% [95% CI 3.6% to 4.9%; $P<0.05$]; 2-year ASRSR, 2000 to 2005, 14% [95% CI 7.8% to 20.6%; $P<0.05$], 5-year ASRSR, 2000 to 2004, 13.4% [95% CI 0.0% to 28.6%; $P<0.05$] (Figure 3.4B). While 2- and 5-year ASRSR only displayed significantly increasing survival trends prior to 2005, 1-year ASRSR showed a consistent increasing trend from 2000-2009.

Given the apparent stagnation in survival rate following more recent years of diagnosis we performed a Kaplan-Meier analysis of different treatment eras (2000-2003 pre-Stupp; 2005-2008 post-Stupp; 2010-2012 targeted therapy; 2014-2016 recent cases) (Figure 3.4C). There was a significant increase in survival from the pre-Stupp period compared to all other eras ($P<0.0001$). Similarly, the Kaplan-Meier analysis also showed improved survival from the post-Stupp era for both the targeted therapy era and more recent cases. ($P<0.0001$), however there was no difference in survival comparing Kaplan-Meier curves for 2010 to 2012 and 2014 to 2016.

Survival Analysis of long-term survivors

To investigate trends in long-term survival outcomes we first determined the appropriateness of Kaplan-Meier and cox regression to detect changes in this cohort. Long-term survivors

were defined as patients surviving more than 2 years (Age-Standardised Relative Survival Rate 19.5% [95% CI, 19.1 to 20%]) (Supplementary Table 3.5). The Kaplan-Meier analysis is a non-parametric test that does not account for the distribution of the data and is weighted towards the majority of cases for which the ~20% of long-term survivors may be under-represented. Similarly, studies have shown that glioblastoma survival violates the proportional hazards assumption suggesting that overall risk of death varies over time from diagnosis.^{218,219} We also confirm this violation in the SEER data, demonstrating that the relative hazard rate increases from time of diagnosis with maximal hazard rates observed around 18 months, following which the hazard rate decreases below initial diagnostic risk levels and continues to decline with time since diagnosis (Figure 3.5A). To account for the difference in hazard risks across time since diagnosis, we isolated long-term survivors for further analysis (Figure 3.5B). In these long-term survivors the age-adjusted hazards ratio increased slightly from 2001 to 2016 of 1.0% per year (95% CI, 0.1 to 1.9; $P < 0.05$) (Figure 3.5C). Analysis of the age-standardised 1-year conditional survival rate showed increase survival trends at 12 months (from 2000 to 2005, 11.1% increase per year [95% CI, 6.4 to 16; $P < 0.05$]) and a decreased trend for survival at 48 months (from 2000 to 2011, -1.6% decrease per year [95% CI, -2.6 to -0.6; $P < 0.05$]) (Figure 3.5D). There was no change in the age-standardised 1-year conditional survival for patients surviving 24 or 36 months from 2000 to 2016. Kaplan-Meier analysis of different treatment periods (2000 to 2003; 2005 to 2008; 2010 to 2012) did not show any difference in survival for long-term survivors (Figure 3.5E; $P = 0.14$). The time period of 2014-2016 was not included in the Kaplan-Meier analysis due to insufficient number of cases with 2-year or more survival data.

Prognostic Factors for long-term survivors

Having identified differences in survival outcomes for long-term survivors, we next investigated whether known glioblastoma prognostic factors were still applicable in this cohort. The results of multivariate analysis for prognostic factors are detailed in Table 3.1 for

glioblastoma cases diagnosed between 2000 to 2016. Year of diagnosis [continuous] associated with an improved hazard ratio over time for all glioblastoma cases (HR, 0.99 [95% CI, 0.99 to 0.99; $P<0.0001$]) but this association was not found in long-term survivors (HR, 1.0; $P=0.345$). Increasing age was associated with worse survival outcome for all cases and also the long-term survivors (HR, 1.03 & 1.02 respectively [95% CI, 1.03 to 1.03 & 1.02 to 1.02]; $P<0.0001$). All types of surgery were associated with reduced hazard ratio for all cases ([HR, 95% CI; P value]. Biopsy [0.63, 0.61 to 0.65; $P<0.0001$], Subtotal Resection [0.68, 0.65 to 0.71; $P<0.0001$], Total Resection [0.49, 0.48 to 0.51; $P<0.0001$]). In long-term survivors only biopsy and total resection significantly associated with a reduced hazard ratio ([HR, 95% CI; P value]. Biopsy [0.85, 0.74 to 0.97; $P=0.013$], Subtotal Resection [0.89, 0.77 to 1.02; $P=0.099$], Total Resection [0.81, 0.71 to 0.92; $P=0.001$]). External Beam Radiation (HR, 0.54 [95% CI, 0.52 to 0.56; $P<0.0001$]) and Chemotherapy (HR, 0.61 [95% CI, 0.59 to 0.63; $P<0.0001$]) all associated with better survival when all diagnosed cases were considered. Radiotherapy and Chemotherapy, however, did not associated with survival outcome in the long-term survivor cohort ([HR; P value] Radiotherapy [1.12; $P=0.12$]; Chemotherapy [1.08; $P=0.18$].

Evaluating survival with best standard of care

Since many patients receive less than the standard of care, we isolated patients that had received surgery, radiotherapy and chemotherapy to determine the survival trends in patients receiving the best standard of care diagnosed between 2000 to 2016. The year 2000 was used as reference for age-adjusted hazards ratio over time. From 2000 to 2016 there is an overall decrease trend in the hazard ratio, with the most significant decrease observed between the years 2000 to 2004, prior to approval of the Stupp protocol (Figure 3.6A). The hazards ratio dropped by -8.6% per year from 2001-2004 (95% CI, -11.6 to -5.6%; $P<0.05$) and decreased further by -2.9% from 2004 to 2016 (95% CI, -3.4 to -2.3%; $P<0.05$). There was no difference in the hazard ratio from 2000 to 2016 when comparing the long-term survivors

(Figure 3.6B). Kaplan-Meier analysis of time periods following the introduction of the Stupp protocol (2005 to 2008 and 2010 to 2016) show no difference in survival curves ($P=0.40$) (Figure 3.6C). Similarly, comparison of long-term survivors during the same period also show no improvement between the periods, though there is a slight insignificant trend towards worse survival outcome in the 2010 to 2016 time period ($P=0.081$) (Figure 3.6D).

To investigate whether the type of chemotherapy used may have impacted survival we used The Cancer Genome Atlas to compare retrospective records of nitrosourea-based (extensively used pre-2005) and temozolomide (the current standard of care) treatments. The cohort used for analysis were glioblastoma ICD-0-3 9440/3 cases diagnosed between 2009 to 2015. Kaplan Meier analysis found no difference between survival curves for nitrosourea-based or temozolomide treatments (Figure 3.6E).

Survival Analysis (cohort diagnosed 2010 to 2016)

While we have reported improved survival trends observed from 2000 to 2009, there is clear stagnation of survival outcomes following this period, representing the most recently diagnosed and relevant glioblastoma cases. We therefore sought to investigate the effects of the current standard of care in this more recent cohort. We selected patients diagnosed between 2010 to 2016 ($N=16,174$) for which the clinical characteristics are detailed in Supplementary Table 3.6.

We performed a multivariate analysis to identify prognostic factors for all glioblastoma cases and long-term survivors diagnosed between 2010 to 2016 (Table 3.2). Year of diagnosis [continuous] did not associate with survival outcome for either all cases (HR, 1.0; $P=0.98$) or long-term survivors (HR, 1.03; $P=0.246$). Increasing age was associated with worse survival outcome for all cases and also long-term survivors (HR, 1.03 & 1.02 respectively [95% CI, 1.03 to 1.03 & 1.02 to 1.02]; $P<0.0001$). All types of surgery were associated with a reduced hazard ratio for all cases ([HR, 95% CI; P value]. Biopsy [0.65, 0.62 to 0.68; $P<0.0001$],

Subtotal Resection [0.63, 0.57 to 0.69; $P<0.0001$], Total Resection [0.48, 0.45 to 0.50; $P<0.0001$]). In long-term survivors only total resection significantly associated with a reduced hazard ratio ([HR, 95% CI; P value]. Biopsy [0.84, 0.67 to 1.04; $P=0.105$], Subtotal Resection [0.84, 0.60 to 1.16; $P=0.291$], Total Resection [0.78, 0.63 to 0.97; $P=0.026$). External Beam Radiation (HR, 0.55 [95% CI, 0.52 to 0.58; $P<0.0001$]) and Chemotherapy (HR, 0.53 [95% CI, 0.50 to 0.56; $P<0.0001$]) all associated with better survival when all diagnosed cases were considered. Radiotherapy and Chemotherapy, however, did not associate with survival outcome in the long-term survivor cohort with Chemotherapy trending towards worse survival outcome, though this was not statistically significant. ([HR, 95% CI; P value] Radiotherapy [0.9, 0.68 to 1.19; $P=0.46$]; Chemotherapy [1.27, 0.97 to 1.66; $P=0.079$].

Evaluating treatment contribution to survival

To determine the overall contribution of each treatment to glioblastoma survival we stratified patients based on treatment status and analysed conditional survival probability in addition to modelling the relative hazard rate based on treatment status.

Surgery

Age-standardised 1-year conditional survival rates demonstrate decreased survival initially at diagnosis for patients who do not receive any form of surgery (Figure 3.7A). Following 12 months, the 1-year conditional survival is similar between patients that receive or do not receive surgery with conditional probability curves overlapping following 24 months of survival from diagnosis and both patients maintain similar survival outcomes. The relative hazard rates for patients receiving surgery start with a lower risk of death that exponentially increases up until ~ 18 months following which the risk of death again decreases eventually reducing to levels lower than the hazard risk at initial diagnosis (Figure 3.7B). Patients that do not receive surgery exhibit a high relative hazard rate at time of diagnosis that decreases

dramatically with survival time during the first 36 months (Figure 3.7C). Comparing the cumulative log hazard rate for surgery treatment status shows overall increased hazard rate from time of diagnosis to 5 years survival compared with patients receiving surgery (Figure 3.7D). Extrapolating this variable hazard rate to a survival probability model also demonstrates decreased survival probability in the no surgery cohort when considering all cases from initial time of diagnosis (Figure 3.7E). In long-term survivors the overall cumulative log hazard rate is similar between surgery and non-surgery groups, which corresponds to a similar survival probability (Figure 3.7F&G).

Radiotherapy

The age-standardised 1-year conditional survival rate for patients receiving or not receiving radiotherapy, also demonstrate a survival advantage at time of diagnosis in the cohort receiving radiotherapy. This survival advantage, however, disappears following 12 months survival and 1-year conditional survival rates remain similar for patients receiving or not receiving radiotherapy for up to 48 months (Figure 3.8A). The relative hazard rate for patients receiving radiotherapy is lowest at time of diagnosis and increases until ~ 20 months whereby the hazard rate decreases over the next 40 months (Figure 3.8B). Patients who did not receive radiotherapy exhibit a high hazards rate at time of diagnosis that exponentially decreases over time over 5 years (Figure 3.8C). Despite the differences in the relative hazard ratio our cumulative log hazards rate shows no significant difference in the overall risk of death between radiotherapy and no radiotherapy groups, which also translates to a lack of survival in our survival probability model (Figure 8D&E)). It should be noted, however, that there is an apparent separation of survival curves in favour of radiotherapy for the first 18 months. Long-term survivors also show similar cumulative log hazard rates and survival probability between radiotherapy and no radiotherapy groups (Figure 8F&G).

Chemotherapy

The age-standardised 1-year conditional survival rate for patients receiving or not receiving chemotherapy, exhibited similar trends to radiotherapy demonstrating a survival advantage at time of diagnosis in the cohort receiving chemotherapy, which fades at around 12-month survival (Figure 3.9A). The relative hazard rate for patients receiving chemotherapy also starts with the lowest risk at diagnosis and increases to the highest risk at ~20 months following which the risk decreases again over the next 40 months (Figure 3.9B). Patients that did not receive chemotherapy also have the highest hazard risk at time of diagnosis which exponentially decreases over the next 5 years (Figure 3.9C). Cumulative log hazard rate of the groups demonstrates an increased hazard rate in the no chemotherapy group, which translates to a decreased probability of survival (Figure 3.9D&E). In long-term survivors, the cumulative log hazard rate for chemotherapy and no chemotherapy groups are similar, though the chemotherapy group trends higher in overall risk though insignificant (Figure 3.9F), which was also observed in the modelled survival probability (Figure 3.9G).

Complete standard of care

Having identified a short-term benefit for the complete standard of care, we next investigated whether patients receiving no treatment had a similar survival outcome for long-term survivors compared to those receiving the best therapeutic care available. We therefore selected for a cohort that were listed as having no record for any form of treatment. The clinical characteristics of these cohorts are listed in Supplementary Table 3.7.

Age-standardised 1-year conditional probability demonstrate a poor survival rate of 6.4% (95% CI, 4.8 to 8.4%) in patients not receiving treatment compared 63.3% (95% CI, 62.2 to 64.3%) in patients receiving the full standard of care (Figure 3.10A). This conditional survival advantage, however, disappears after 12 months, with many patients reporting higher conditional survival probabilities in the no treatment group compared to those receiving the full standard of care. The relative hazard rate for patients receiving the complete standard of care has the lowest hazard rate at time of diagnosis, increasing until ~20 months, following

which the hazards risk decreases over the next 20 months (Figure 3.10B). In comparison the no treatment cohort display a disproportionately high relative hazard rate at the time of diagnosis, which drastically reduces over the first 12 month (Figure 3.10C). The cumulative log hazard rate also demonstrates a significant increase in the hazards rate for patients in the not treated group, although the cumulative hazard rate continues to increase over time for the complete standard of care whereas the no treatment group has a lower rate of increase leading to similar cumulative hazard risk at 5 years survival (Figure 3.10D). Extrapolating the cumulative log hazard to overall survival demonstrates the significant difference in short-term survival with the complete standard of care providing the best survival outcome (Figure 3.10E). In long-term survivors the cumulative log hazard risk between treated and non-treated groups is similar with no significant difference in the survival probabilities (Figure 3.10F&G).

Prognostic factors for long-term survivors by age group

Long-term survivors presented in the SEER database associate with younger age, which is a major independent factor for survival and even treatment selection among glioblastoma cases. To determine if the treatment associations reported in this study are in part due to age-group selection, we stratified patients diagnosed between 2010 to 2016 into 3 age-group brackets (20-44, 45-64, 65-85+ [clinical characteristics are presented in Supplementary Table 3.8]) and performed multivariate analysis on all cases and long-term survivors (Table 3.3).

Age, Surgery, Radiotherapy and Chemotherapy all associated with improved survival outcome for all age-groups when all glioblastoma cases were considered. Year of diagnosis [continuous] associated with a reduced hazard ratio only in patients 65 years old above (HR, 0.98, 95% CI, 0.97 to 1.0; P=0.033). In long-term survivors, age remained as a statistically significant prognostic factor in patients 65 years old and above associating with worse survival (HR, 1.03, 95% CI 1.0 to 1.04; P=0.049). No other prognostic factors significantly associated with long-term survivors in any of the age-groups.

Ages 20-44

Age [continuous] (HR, 1.03; $P<0.0001$), Surgery ([HR; P value]. Biopsy [0.71; $P=0.002$], Subtotal Resection [0.61; $P=0.02$], Total Resection [0.55; $P<0.0001$]), External Beam Radiation (HR, 0.62; $P<0.001$) and Chemotherapy (HR, 0.68; $P<0.003$) associated with better survival when all diagnosed cases were considered. The year of diagnosis did not associate with improved survival in this cohort ($P=0.262$). No variables associated with survival in the long-term survivor cohort.

Ages 45-64

Age [continuous] (HR, 1.03; $P<0.0001$), Surgery ([HR; P value]. Biopsy [0.63; $P<0.0001$], Subtotal Resection [0.61; $P<0.0001$], Total Resection [0.47; $P<0.0001$]), External Beam Radiation (HR, 0.58; $P<0.0001$) and Chemotherapy (HR, 0.55; $P<0.0001$) associated with better survival when all diagnosed cases were considered. The year of diagnosis did not associate with improved survival in this cohort ($P=0.201$). No variables associated with survival in the long-term survivor cohort.

Ages 65-85+

Year of Diagnosis [continuous] (HR, 0.98; $P<0.033$), Age [continuous] (HR, 1.03; $P<0.0001$), Surgery ([HR; P value]. Biopsy [0.66; $P<0.0001$], Subtotal Resection [0.65; $P<0.0001$], Total Resection [0.48; $P<0.0001$]), External Beam Radiation (HR, 0.52; $P<0.0001$) and Chemotherapy (HR, 0.51; $P<0.0001$) associated with better survival when all diagnosed cases were considered. Only Age remained as prognostic factor in the long-term survivor cohort (HR, 1.02; $P=0.049$).

3.5 Discussion

The Stupp protocol remains the most significant and only advancement to the therapeutic care for glioblastoma in the past two decades. Following the introduction of the Stupp protocol in 2005 early registry data has sought to confirm the survival improvement of the Stupp protocol by comparing time periods pre- and post- Stupp, concluding improved survival outcomes.^{112,113} Our study questions the fundamental assumption that improvement in glioblastoma survival is mainly attributed to the Stupp protocol. In this population-wide registry data analysis we report that glioblastoma survival improved most significantly in the years prior to the introduction of the Stupp protocol between 2000 to 2005. While a smaller trend in improvement was observed following the introduction of the Stupp protocol, our results clearly demonstrate that survival is not static over time and that Stupp independent factors have improved glioblastoma prognosis.

While this study demonstrates an overall improvement to survival over the years, these benefits diminish with each year of survival with 2- and 5-year survival rates remaining relatively unchanged since the introduction of the Stupp protocol. Furthermore, our analysis of long-term survivors representing ~20% of all glioblastoma cases, have shown no survival improvement since the year 2000. This is consistent with a recent US population-wide registry study that also reported unchanged 5-year survival rates with the introduction of the Stupp protocol.¹¹⁶ These results conflict with the 5-year survival rate increase of 1.9% to 9.8%, reported in the original EORTC-NCIC phase III clinical trial. Together it is clear that there is stagnation in survival outcomes for glioblastoma with the current standard of care contributing little to the overall advancement to therapeutic care.

The lack of general efficacy can be partly explained by patient MGMT methylation status, which is an independent factor for temozolomide response.⁹³ Studies have conclusively demonstrated that temozolomide treatment is ineffective in patients with unmethylated MGMT promoter, yet MGMT biomarker assessment is not used in clinical decision making,

with the exception of elderly glioblastoma (aged 65 years or more).^{93,98,110} Concerningly the majority of glioblastoma cases present with unmethylated MGMT promoter with only 30-45% exhibiting promoter methylation.^{93,99,100} Furthermore, a recent study by Brennan and colleagues suggest that MGMT promoter methylation is only prognostically relevant in glioblastoma classical subtype tumours, with proneural and mesenchymal demonstrating no survival difference based on MGMT status.¹⁷ With increasing studies appreciating the cellular plasticity of glioblastoma cells leading to cellular subtype switching, these findings may contribute to the overall variation and resistance observed in the general population.^{25,35}

Due to the relatively young age of patients enrolled in the EORTC-NCIC clinical trial, the Stupp protocol was not directly translated into a standard of care for elderly glioblastoma patients, who represent 50% of all glioblastoma cases.^{7,91,105} It was not until 2012 that the NOA-08 and Nordic phase III clinical trial demonstrated non-inferiority of temozolomide compared to hypofractionated radiotherapy for elderly glioblastoma.^{107,108} This survival benefit was restricted to MGMT promoter methylated tumours. Subsequently the use of temozolomide in elderly glioblastoma was only recently accepted as an alternate standard of care with MGMT promoter methylation patients.¹¹⁰ Our study, however, did not demonstrate any improvement in survival rates following the introduction of this care in 2012. Recently in 2017, an EORTC-NCIC CTG phase III clinical trial investigated a modified chemoradiation strategy for elderly glioblastoma, demonstrating improved survival compared to hypofractionated radiotherapy-alone.¹⁰⁹ This survival improvement was again restricted to MGMT promoter methylated tumours. Since this alternate regime has only recently been introduced, our study was unable to determine its impact on the general population.

Recent studies have demonstrated that glioblastoma survival has a nonconstant hazard of death over time.^{218,219} We therefore hypothesised that response to treatment may differ between short- and long- term survivors. Indeed, our results confirm that the survival benefit provided by surgery, radiotherapy and chemotherapy only persisted for 18-20 months from

initial diagnosis. Following this time-period, the conditional probability of long-term survival was consistent even among patients that did not receive any form of treatment. Prognostically only age and total resection associated with reduced risk of death in the long-term survivor cohort. This association was observed in all age-groups, suggesting that it is a common feature of glioblastoma biology. From these results we propose that the current standard of care improves short-term survival, most likely associated with primary tumour control, but disease progression in long-term survival is unique and not targeted by the current treatments, which has led to a stagnation in survival outcomes.

Unlike other solid malignancies where significant advances have been made through clinical trials, the clinical trial success rate for objective improvement in patient survival is almost 0%, with temozolomide the only successful agent to translate to clinical care.²⁰⁸ The fundamental misalignment with clinical trial development and ultimate success may lie in our incomplete understanding of glioblastoma pathology. Recently it has been reported that glioblastoma tumourigenesis may occur up to 7 years before diagnosis with the molecular drivers already preordained at time of diagnosis.³⁶ Novel therapeutic treatments therefore need to account for the full virulence and biology of glioblastoma at the time of diagnosis. Unfortunately, we are yet to identify the major factors contributing to overall tumour progression and recurrence which has led to blind clinical trial development, inevitably leading to the clinical stagnation we see today. Early detection may also present new opportunities to target a less developed and aggressive form of cancer, which should be further explored in light of the dismal success of recent clinical trial testing.

It is important to note that while survival in long-term survivors remain unchanged over the time-period of our analysis, relative survival rates for patients receiving the complete standard of care are superior to those that did not. It is possible that receiving complete treatment increases the proportion of patients reaching long-term survivorship, however, patients selected for the full standard of care are also more likely to have favourable

prognostic factors and a lower baseline hazard, making long-term survivorship more likely in this cohort.

There are limitations in the retrospective nature of this study. While the SEER dataset incorporates a large number of well-characterised clinical data points, radiotherapy and chemotherapy records may be incomplete due to patients receiving out-patient treatment. Despite this, SEER reports a high positive predictive value (>85%) for these treatments in other solid cancers and the reported percentages of patients receiving radiotherapy or chemotherapy reported in this study are consistent with other large US population-wide registry datasets for glioblastoma over the same study period.^{385,386} Additionally, the 2016 World Health Organisation Classification of Tumour of the Central Nervous System revisions now classifies IDH1/2 mutation status as a primary diagnostic criteria for glioblastoma.³⁸³ This molecular classification was not available in the current analysis and could not be investigated for its effects on survival outcome.

3.6 Conclusion

In summary, this study has provided evidence of a 20-year clinical stagnation to survival outcomes for glioblastoma with the Stupp protocol providing marginal benefits to short-term survival only. Current research directions are failing to treat the complexity of glioblastoma and an understanding of the tumour biology unpinning late-stage tumour progression or early diagnosis is needed to advance therapeutic care.

Figure Legends

Figure 3.1. Discrepancies in median overall survival reporting for the Stupp protocol across clinical trials.

Clinical data was obtained from phase II and III clinical trials using Pubmed. The search terms glioblastoma, gbm, temozolomide, stupp and clinical trials was used, and articles manually checked for data inclusion. Studies that did not use the Stupp protocol or did not contain median survival data or 95% confidence intervals were excluded. A forest plot of included studies was generated in R software reporting median overall survival and 95% confidence intervals.

Figure 3.2. Glioblastoma age-adjusted incidence rates from 2001 to 2016.

Raw counts were obtained from the NCI SEER and NPCR databases and standardised to the 2010 US census population. Age-adjusted incident rates and annual percentage change (APC) are reported for all glioblastoma cases (A) and Males and Females separately (B). APC was determined using SEER's JoinPoint Regression Program. ** $P < 0.05$ is statistically significant. 95% confidence intervals are reported for all data.

Figure 3.3. Selection of the glioblastoma analytic cohort from the Surveillance, Epidemiology, and End Results Program (SEER) database.

Figure 3.4. Trends in glioblastoma survival show early improvement but progress has recently stagnated. Trends in the overall hazard ratio for glioblastoma from the year 2000 [reference] (A). Trends in the age-standardised relative overall survival rate for glioblastoma patients by year of diagnosis (B). SEER's JoinPoint Regression program was used to determine significant trends in the annual percentage change (APC) of the hazard ratio and relative overall survival. ** refers to $P < 0.05$. Kaplan-Meier survival curves for patients diagnosed in the years 2000 – 2003, 2005 – 2008, 2010 – 2012 and 2014 – 2016 (C). Log-rank test and BH pair-wise multi-comparison test was used to compared between survival

curves. The null hypothesis was rejected when $P < 0.05$. 95% confidence intervals are reported for all data.

Figure 3.5. Trends in long-term survivors have not improved since the year 2000.

Relative hazard rate from time of diagnosis of glioblastoma for patients diagnosed between 2000-2016 (A). Kaplan-Meier survival curve of glioblastoma cases diagnosed between 2000-2016, patients surviving 2 years or longer (long-term survivors) are outlined in the red box (B). Trends in the overall hazard ratio for long-term glioblastoma survivors from the year 2000 [reference] (C). The age-standardised 1-year conditional survival rate of all glioblastoma cases (D). SEER's JoinPoint Regression program was used to determine significant trends in the annual percentage change (APC) of the hazard ratio and 1-year conditional overall survival. ** refers to $P < 0.05$. Kaplan-Meier survival curves for patients diagnosed in the years 2000 – 2003, 2005 – 2008, 2010 – 2012 (E). The Log-rank test was used to compare between survival curves. The null hypothesis was rejected when $P < 0.05$. 95% confidence intervals are reported for all data. Glioblastoma cases diagnosed between 2014 – 2016 were not included due to insufficient available cases reaching 2 years or more.

Figure 3.6. Short-term glioblastoma survival trends have improved in patients receiving the complete standard of care, yet long-term survival remains unchanged.

Trends in the overall hazard ratio for glioblastoma patients receiving the complete standard of care from the year 2000 [reference] (A). Trends in the overall hazard ratio for long-term glioblastoma survivors receiving the complete standard of care from the year 2000 [reference] (B). SEER's JoinPoint Regression program was used to determine significant trends in the annual percentage change (APC) of the hazard ratio. ** refers to $P < 0.05$. Kaplan-Meier survival curves of all glioblastoma cases (C) and long-term survivors (D) receiving the complete standard of care and diagnosed in the years 2005 – 2008 and 2010 – 2016. Kaplan-Meier survival curve for patients in The Cancer Genome Atlas (TCGA) receiving nitrosourea-based or temozolomide chemotherapy (E). The Log-rank test was used to compare between survival

curves. The null hypothesis was rejected when $P < 0.05$. 95% confidence intervals are reported for all data.

Figure 3.7. Surgery improves short-term survival but provides no benefit for long-term survivors in the modern glioblastoma treatment era. All glioblastoma cases diagnosed between the year 2010 – 2016 were selected for analysis. Age-standardised 1-year conditional survival rate for patients receiving surgery or not surgery from time of diagnosis (A). A model of the relative hazard rate for patients receiving surgery (B) and not receiving surgery (C) from time of diagnosis to 5 years. The cumulative log hazard rate (D) and overall survival probabilities (E) were calculated from the models determined in (B) and (C). The relative hazard rate models were recalculated for long-term survivors and used to generate the long-term survivors cumulative log hazard rate (F) and overall survival probabilities (G). 95% confidence intervals are reported for all data.

Figure 3.8. Radiotherapy may not confer a significant survival advantage in current glioblastoma treatment. All glioblastoma cases diagnosed between the year 2010 – 2016 were selected for analysis. Age-standardised 1-year conditional survival rate for patients receiving radiotherapy or not receiving radiotherapy from time of diagnosis (A). A model of the relative hazard rate for patients receiving radiotherapy (B) and not receiving radiotherapy (C) from time of diagnosis to 5 years. The cumulative log hazard rate (D) and overall survival probabilities (E) were calculated from the models determined in (B) and (C). The relative hazard rate models were recalculated for long-term survivors and used to generate the long-term survivors cumulative log hazard rate (F) and overall survival probabilities (G). 95% confidence intervals are reported for all data.

Figure 3.9. Chemotherapy improves short-term survival but provides no benefit for long-term survivors in the modern glioblastoma treatment era. All glioblastoma cases diagnosed between the year 2010 – 2016 were selected for analysis. Age-standardised 1-year conditional survival rate for patients receiving chemotherapy or not receiving chemotherapy

from time of diagnosis (A). A model of the relative hazard rate for patients receiving chemotherapy (B) and not receiving chemotherapy (C) from time of diagnosis to 5 years. The cumulative log hazard rate (D) and overall survival probabilities (E) were calculated from the models determined in (B) and (C). The relative hazard rate models were recalculated for long-term survivors and used to generate the long-term survivors cumulative log hazard rate (F) and overall survival probabilities (G). 95% confidence intervals are reported for all data.

Figure 3.10. Long-term glioblastoma patients who received the complete standard of care or no treatment have similar survival outcomes. All glioblastoma cases diagnosed between the year 2010 – 2016 were selected for analysis. Age-standardised 1-year conditional survival rate for patients receiving the complete standard of care or recorded as not receiving any treatment from time of diagnosis (A). A model of the relative hazard rate for patients receiving the complete standard of care (B) and no treatment (C) from time of diagnosis to 5 years. The cumulative log hazard rate (D) and overall survival probabilities (E) were calculated from the models determined in (B) and (C). The relative hazard rate models were recalculated for long-term survivors and used to generate the long-term survivors cumulative log hazard rate (F) and overall survival probabilities (G). 95% confidence intervals are reported for all data.

Figures and Tables

Figure 3.1

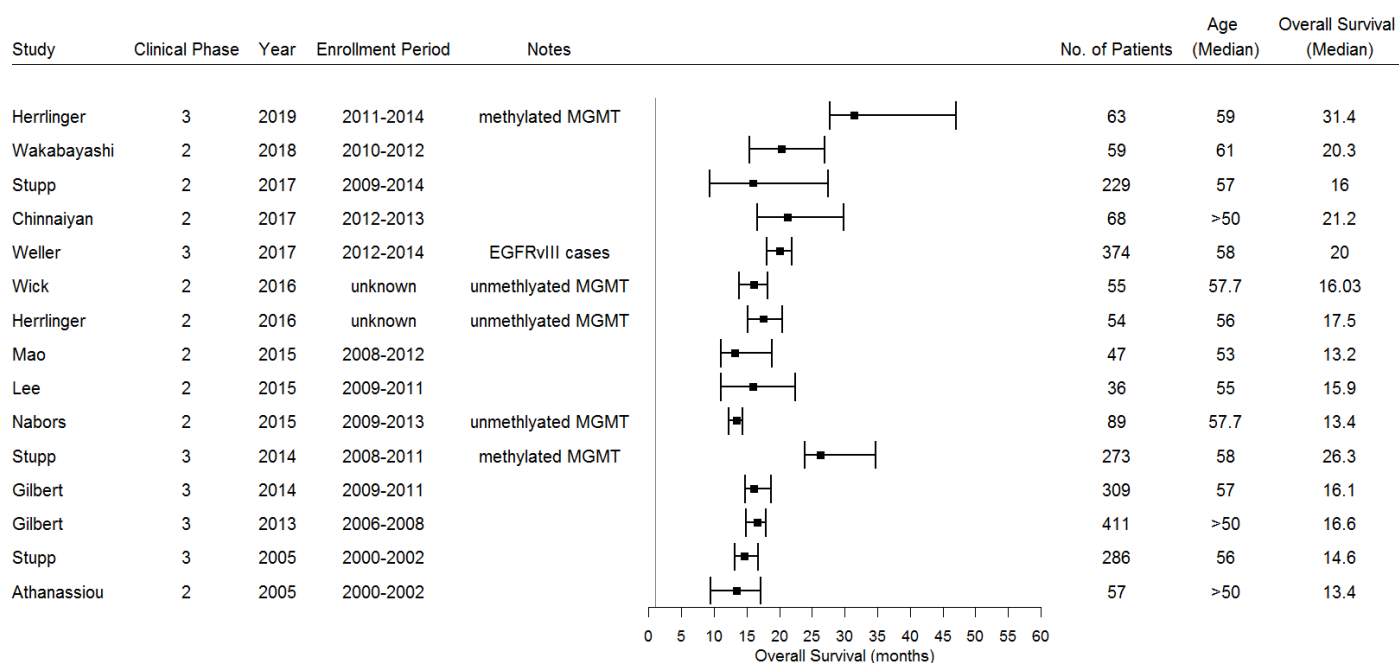
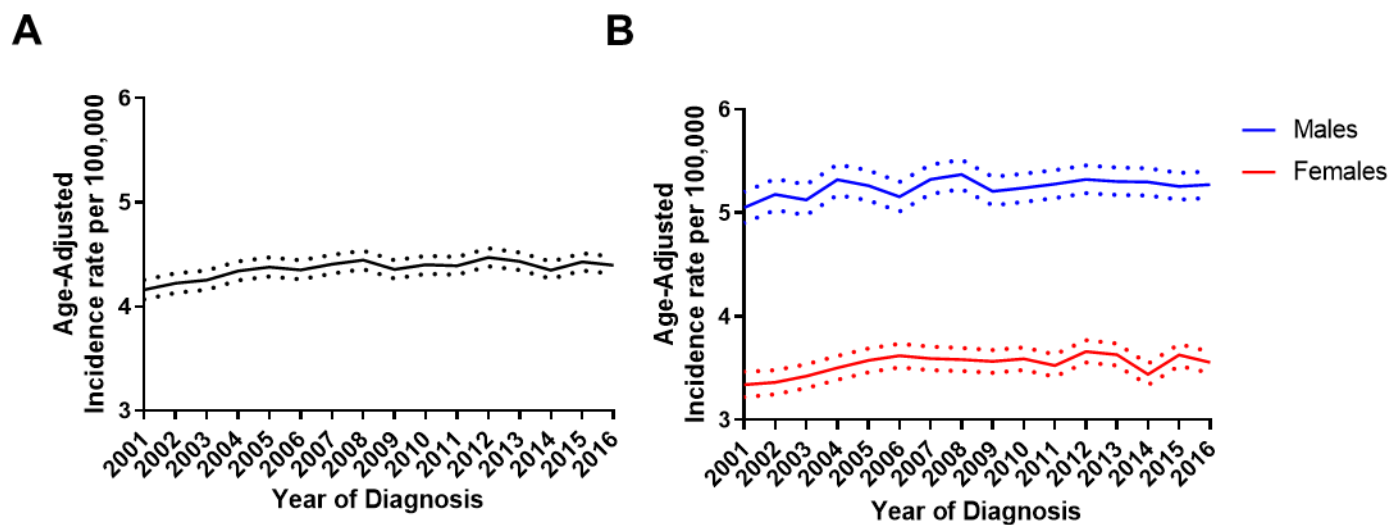


Figure 3.2



	Trend 1		Trend 2	
	Years	APC (95% CI)	Years	APC (95% CI)
Total	2001-2005	1.3 (0.4, 2.3) **	2005-2016	0.1 (-0.1, 0.2)
Males	2001-2016	0.2 (0.0, 2.3) **		
Females	2001-2006	1.6 (0.3, 2.9) **	2006-2016	-0.1 (-0.5, 0.3)

**P<0.05

Figure 3.3

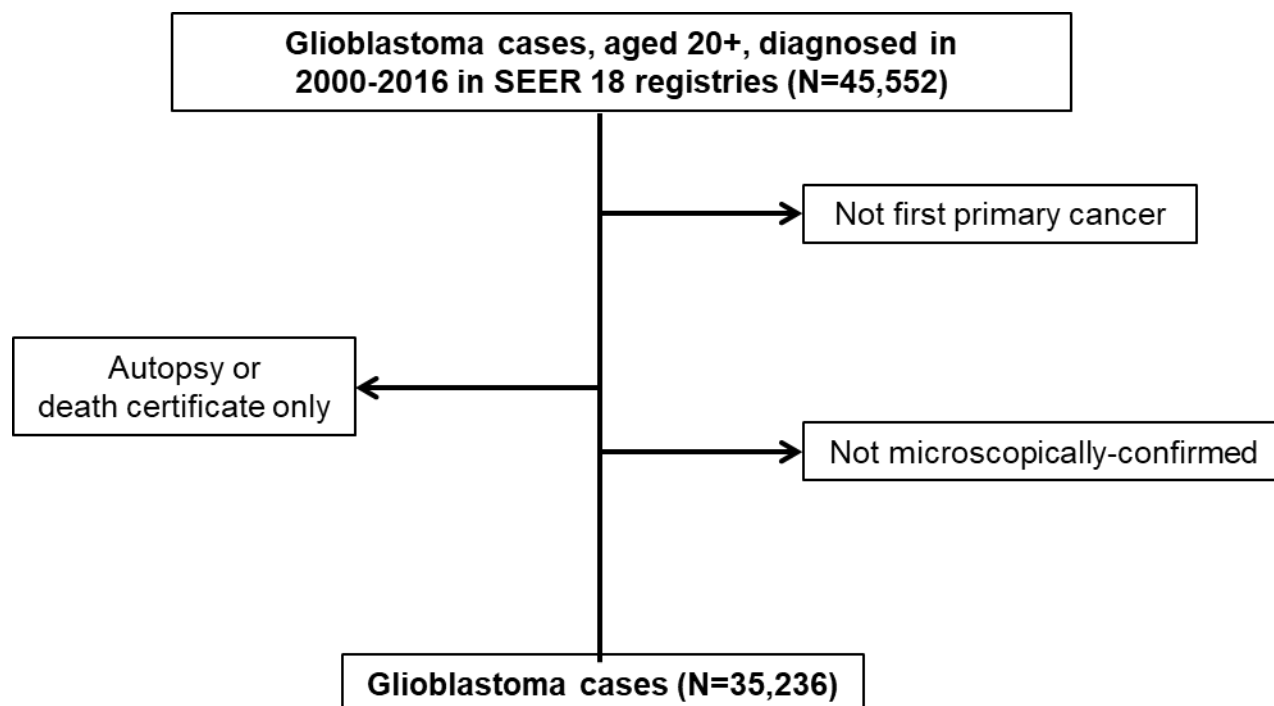
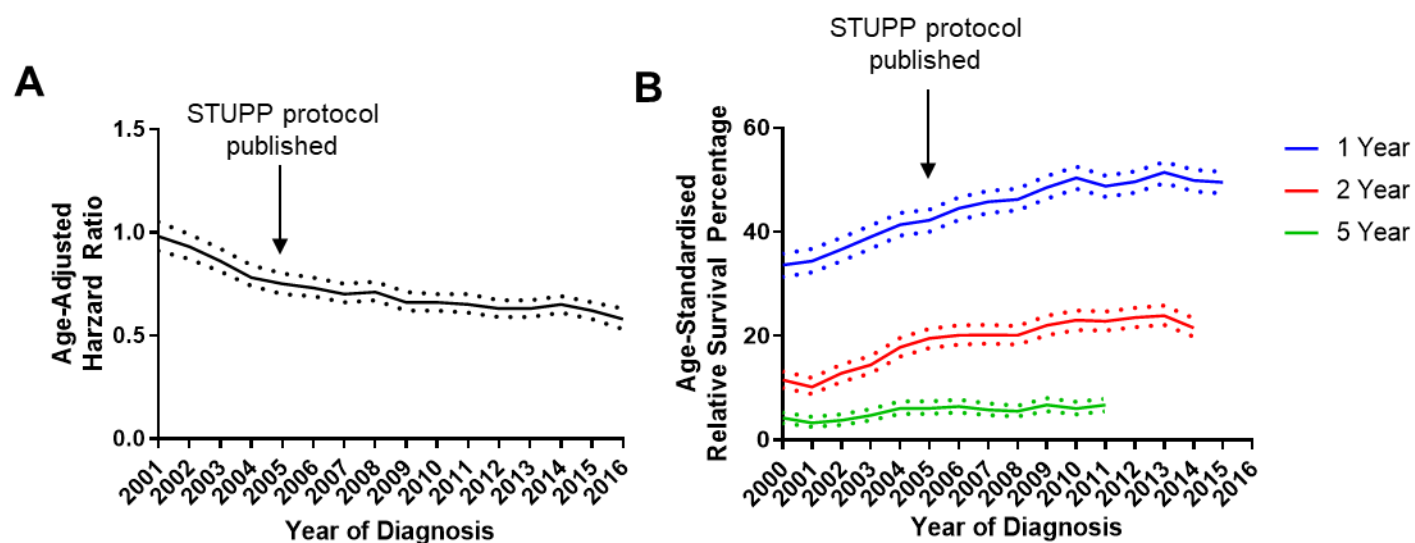


Figure 3.4

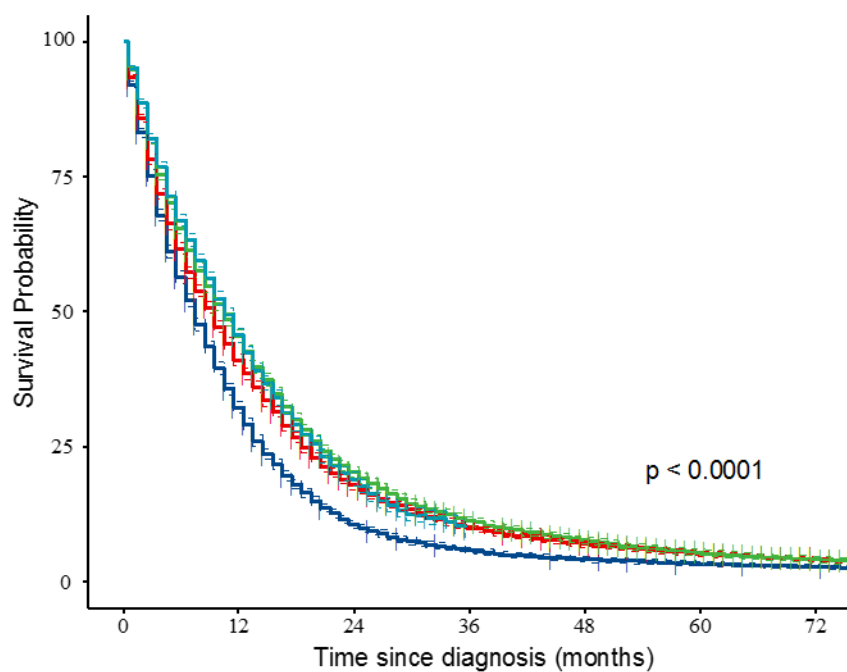


	Trend 1		Trend 2	
	Years	APC (95% CI)	Years	APC (95% CI)
Age-Adjusted Hazard Ratio				
All Patients	2001-2005	-7.1 (-8.8,-5.4)**	2005-2016	-1.9 (-2.4,-1.4)**
Long-Term Survivors	2001-2014	1.0 (0.1,1.9)**		
Age-Standardised Relative Survival				
1 year	2000-2009	4.2 (3.6,4.9)**	2009-2015	0.3 (-0.6,1.2)
2 year	2000-2005	14.0 (7.8,20.6)**	2005-2014	2.1 (0.4,3.7)
5 year	2000-2004	13.4 (0.0,28.6)**	2004-2011	1.6 (-2.0,5.4)

**P<0.05

Figure 3.4 (continued)

C



Year — 2000-2003 — 2005-2008 — 2010-2012 — 2014-2016

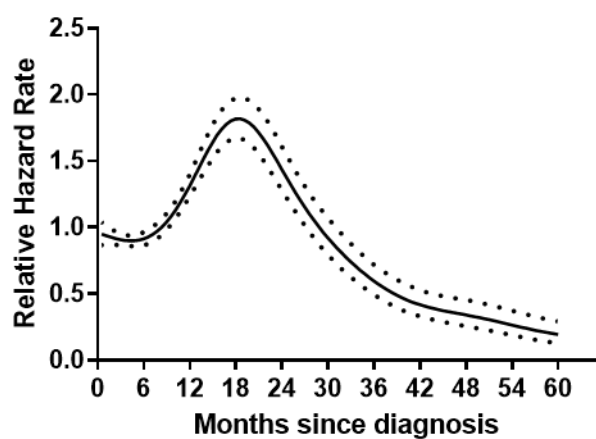
Number at Risk							
	0	12	24	36	48	60	72
2000-2003	7094	2274	738	402	285	226	186
2005-2008	7925	3222	1394	782	526	384	308
2010-2012	6659	2989	1317	719	463	196	69
2014-2016	7189	2071	415	0	0	0	0

Time since diagnosis (months)

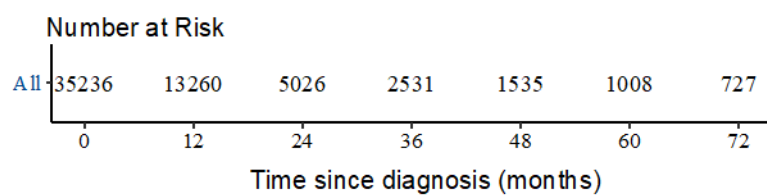
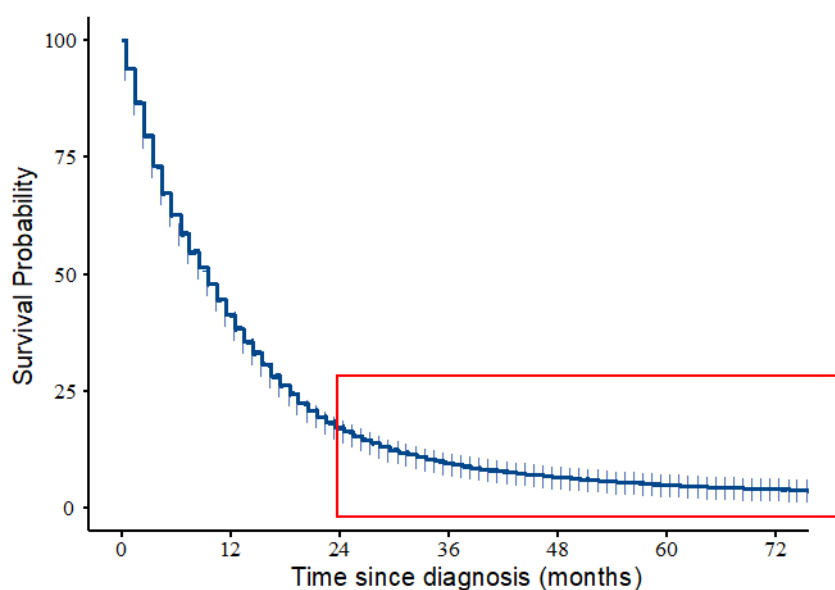
Pair-wise multicomparison (p value)				
Year	2000-2003	2005-2008	2010-2012	2014-2016
2005-2008	<0.0001			
2010-2012	<0.0001	<0.0001		
2014-2016	<0.0001	<0.0001	0.48	

Figure 3.5

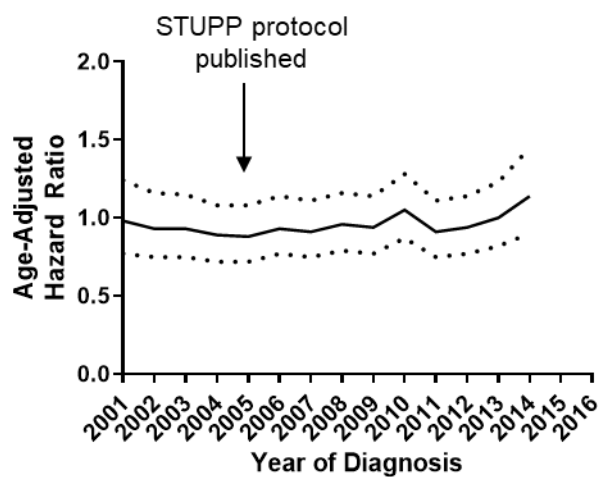
A



B



C



D

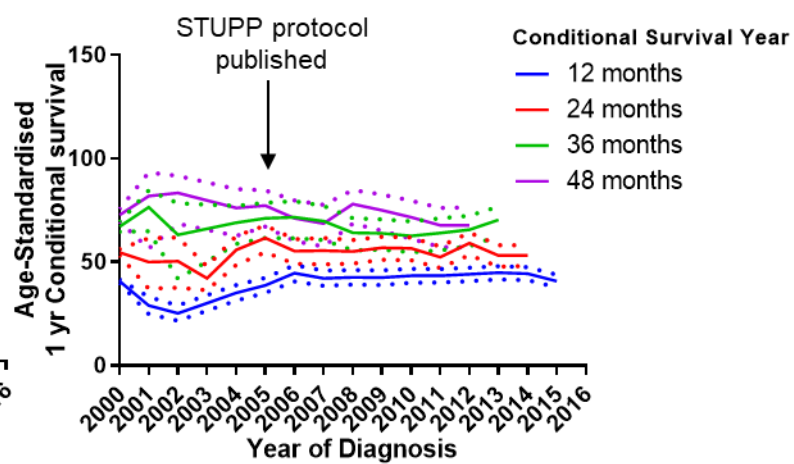


Figure 3.5 (continued)

	Trend 1		Trend 2	
	Years	APC (95% CI)	Years	APC (95% CI)
Age-Adjusted Hazard Ratio	2001-2014	1.0 (0.1,1.9)**		
Age-Standardised 1 year Conditional Survival				
12 months	2000-2005	11.1 (6.4,16)**	2005-2014	0.1 (-1.1,1.3)
24 months	2000-2013	0.3 (-1.0,1.7)		
36 months	2000-2012	-0.9 (-1.9,0.1)		
48 months	2000-2011	-1.6 (-2.6,-0.6)**		

**P<0.05

E

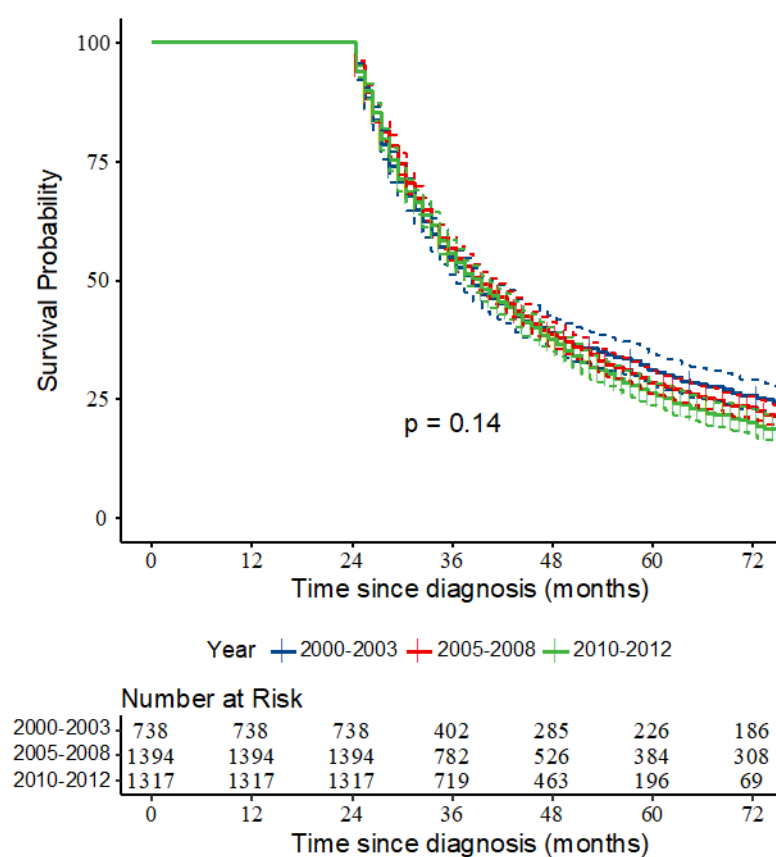
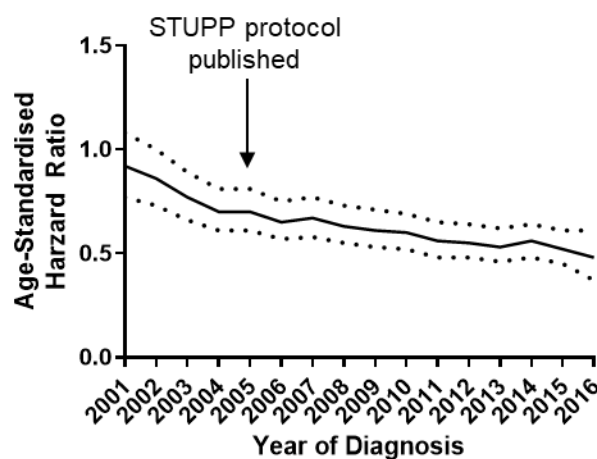
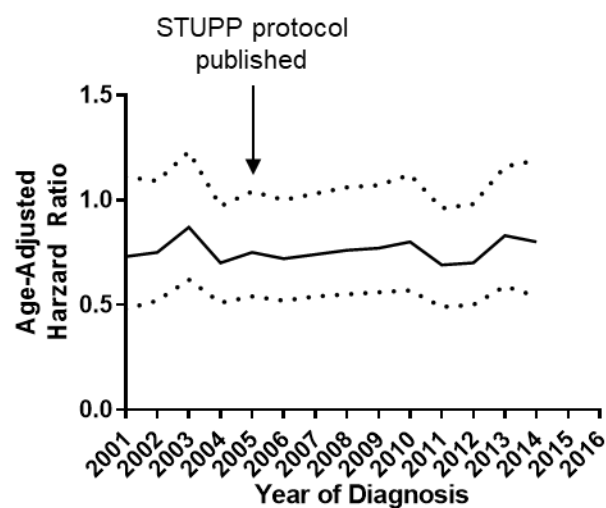


Figure 3.6

A



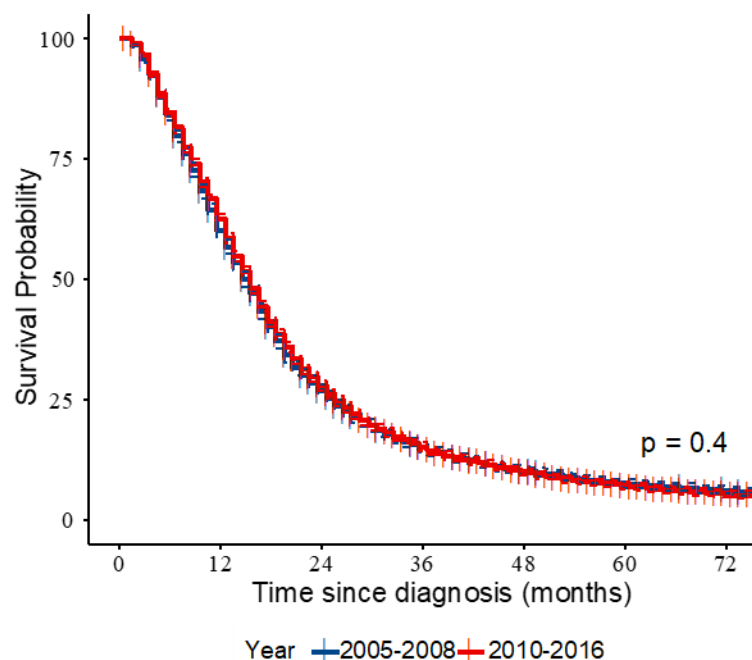
B



	Trend 1		Trend 2	
	Years	APC (95% CI)	Years	APC (95% CI)
Complete Treatment				
All Cases	2001-2004	-8.6 (-11.6,-5.6)**	2004-2016	-2.9 (-3.4,-2.3)**
Long-Term Survivors	2001-2016	0.1 (-1.0,1.2)	-	-

**P<0.05

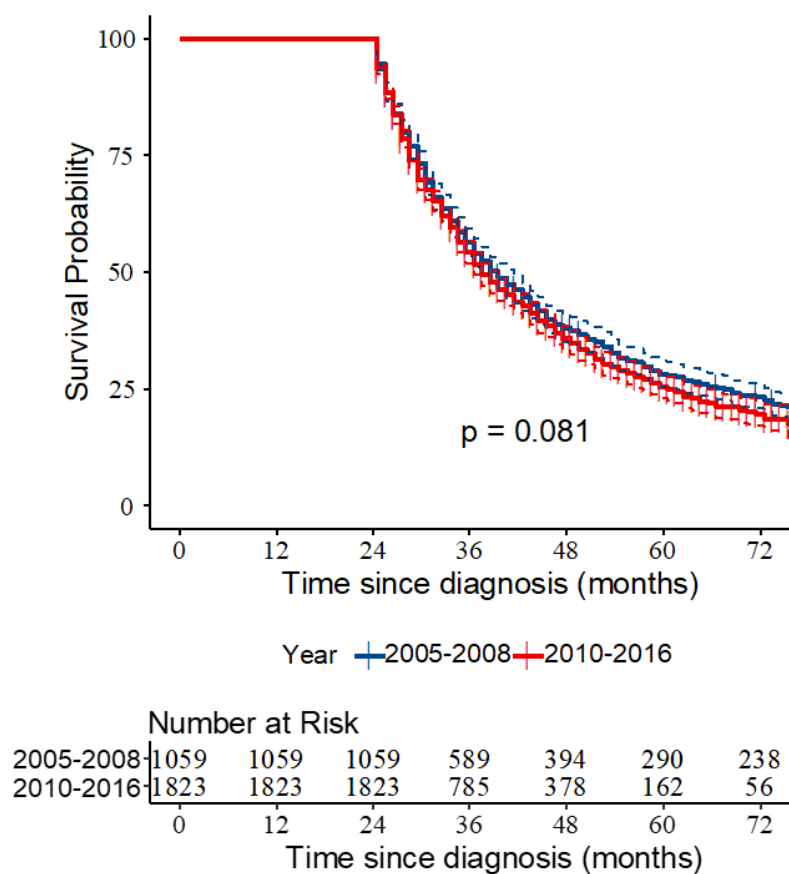
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Number at Risk							
2005-2008	3995	2394	1059	589	394	290	238
2010-2016	9555	4987	1823	785	378	162	56
	0	12	24	36	48	60	72
	Time since diagnosis (months)						

Figure 3.6 (continued)

D



E

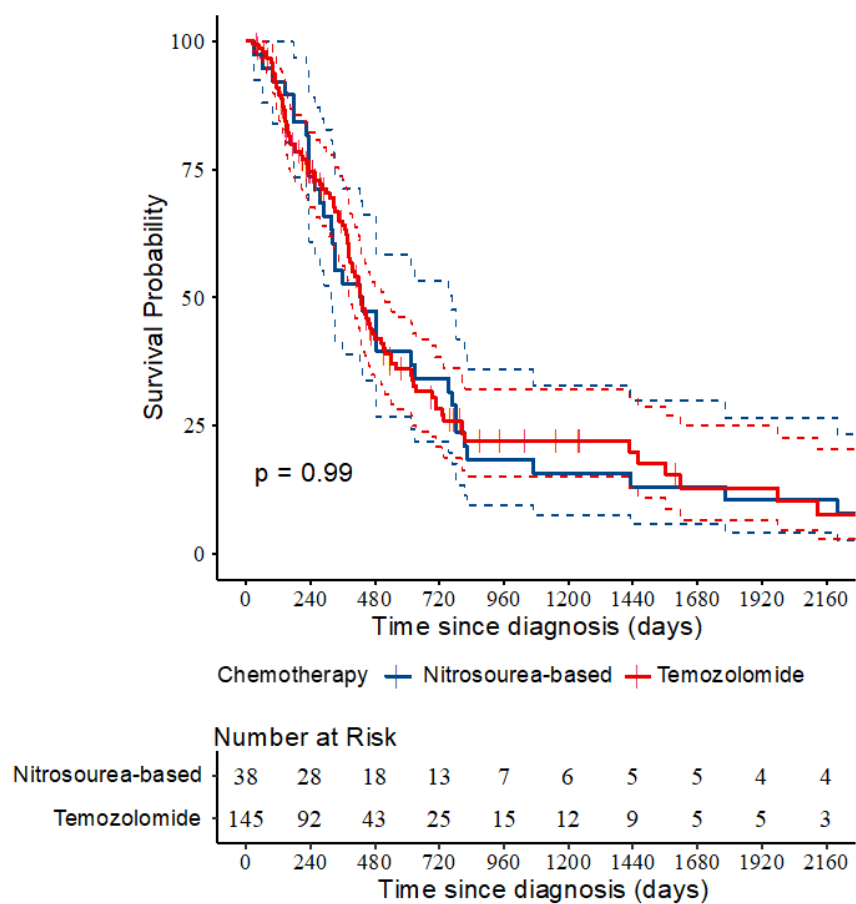


Figure 3.7

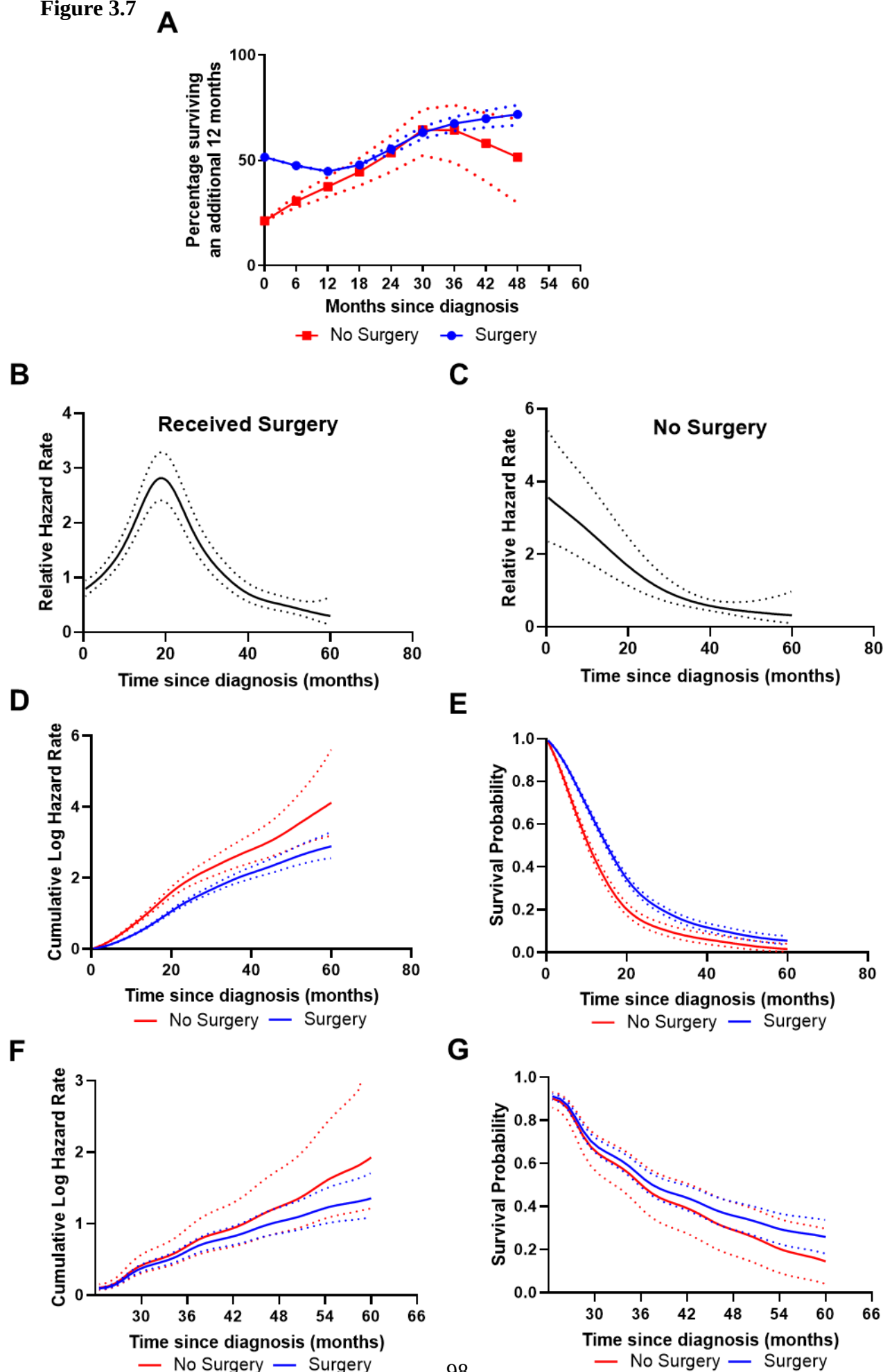


Figure 3.8

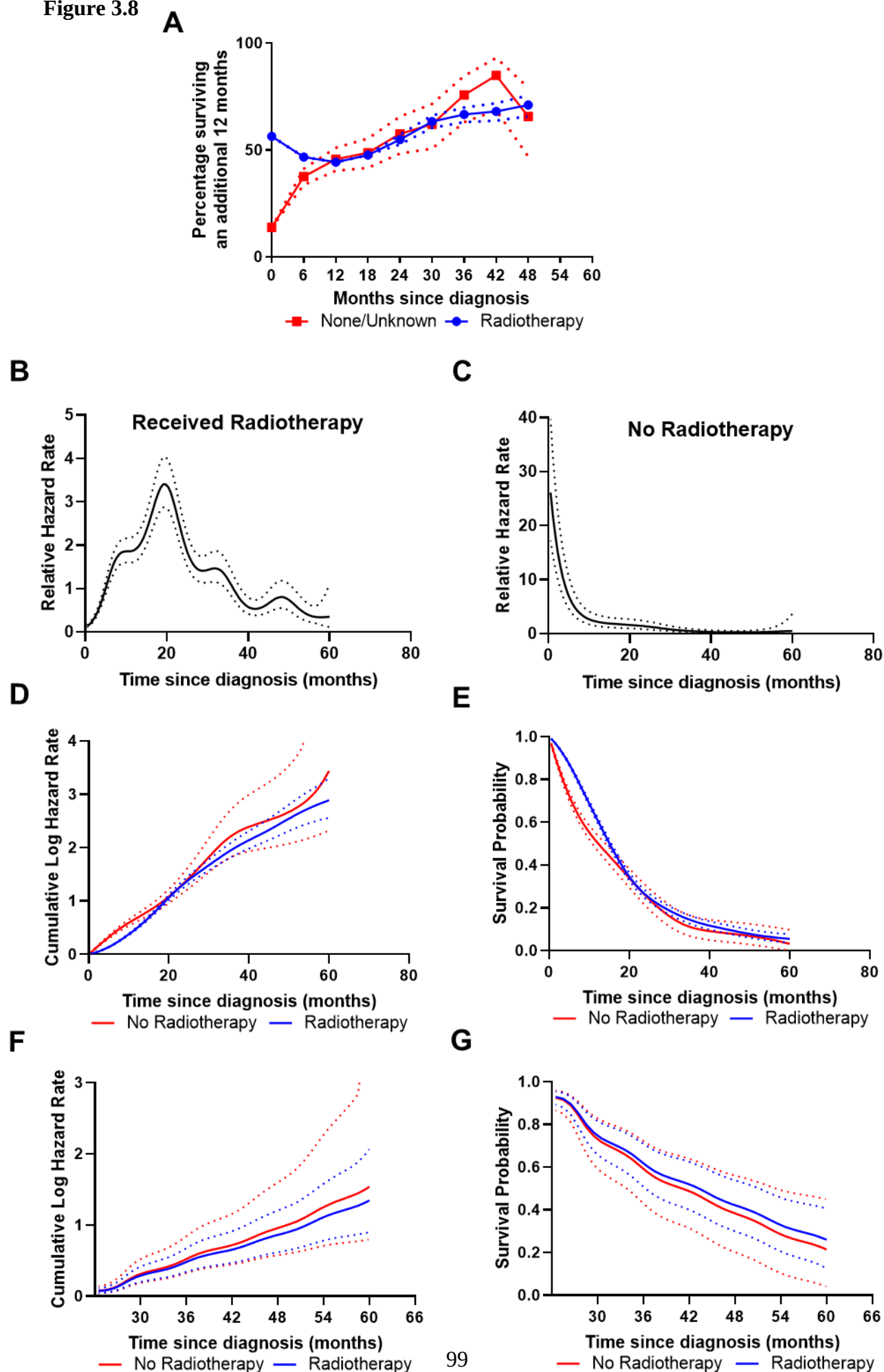
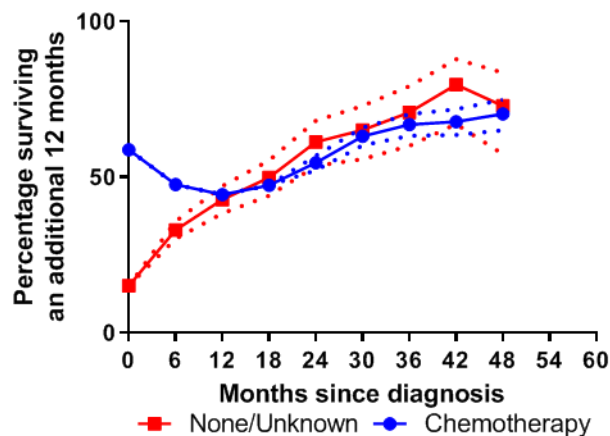
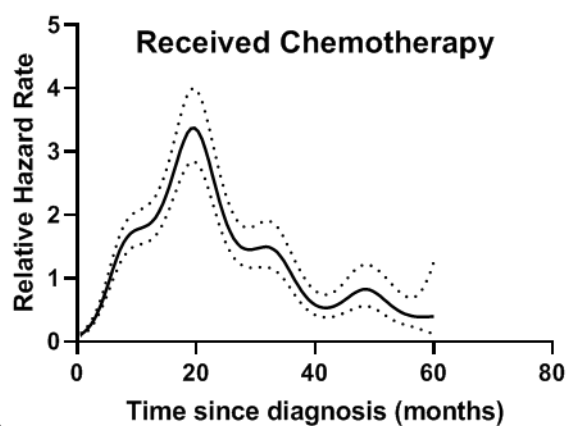


Figure 3.9

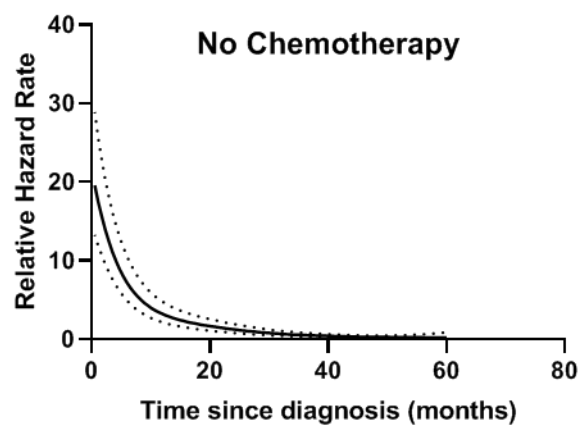
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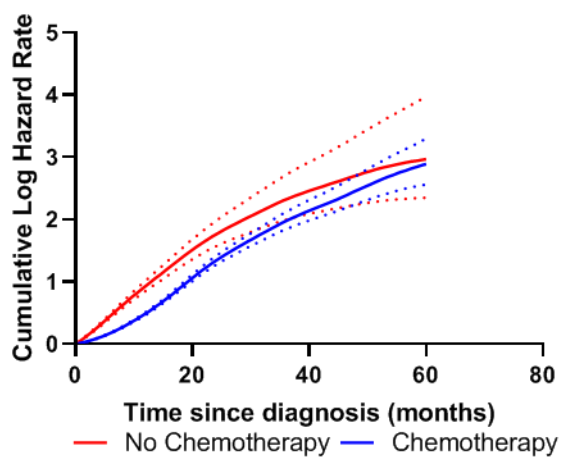
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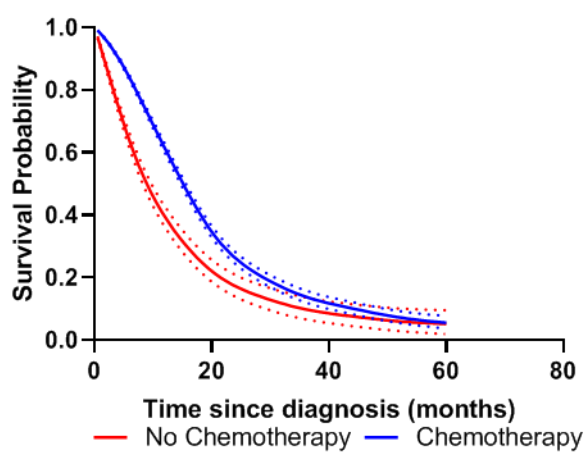
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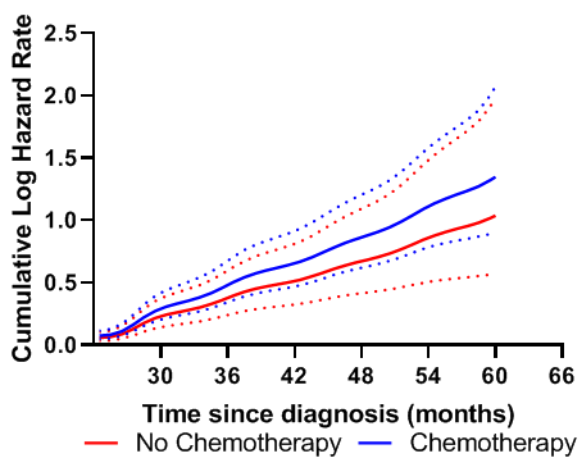
D



E



F



G

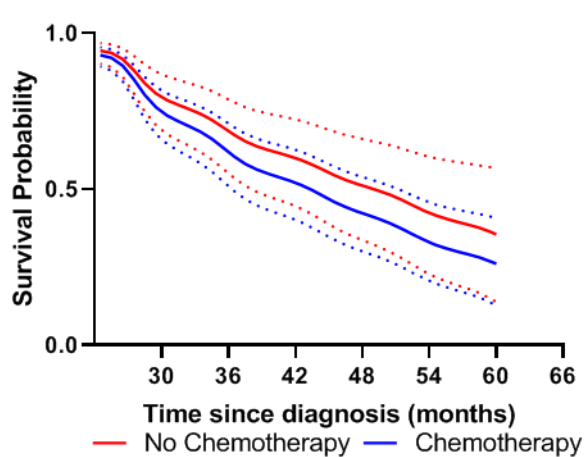
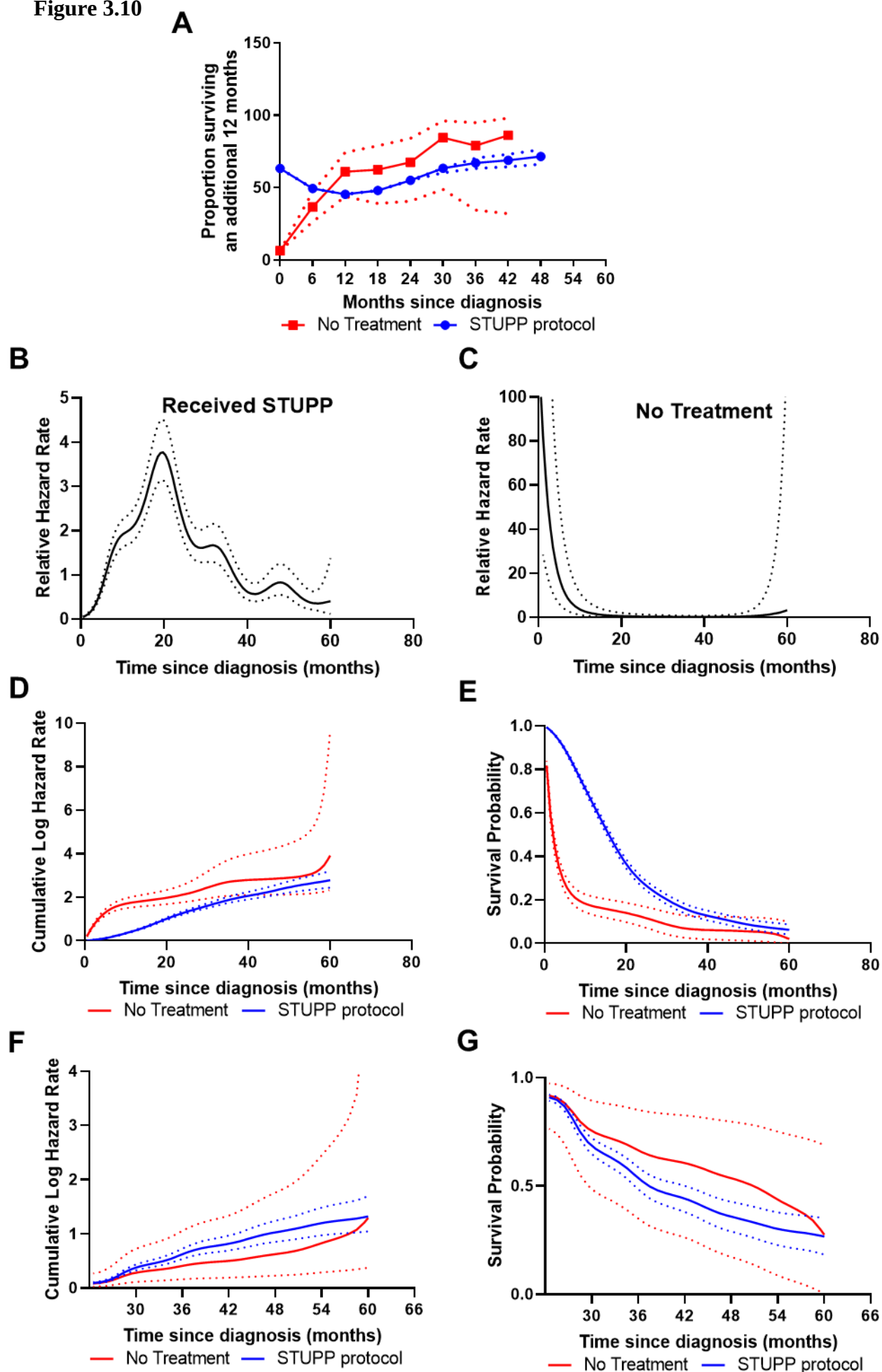


Figure 3.10



Tables

Table 3.1. Multivariate Analysis of known prognostic factors in long-term glioblastoma survivors diagnosed between the years 2000 – 2016.

	Cases from Diagnosis (N = 34,292)				Long-Term Survivors (N=4,859)			
	Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value
Year of Diagnosis	0.99	0.99	0.99	<0.0001	1	0.99	1.01	0.345
Age	1.03	1.03	1.03	<0.0001	1.02	1.02	1.02	<0.0001
Surgery								
No Surgery	Reference				Reference			
Biopsy	0.63	0.61	0.65	<0.0001	0.85	0.74	0.97	0.013
Subtotal	0.68	0.65	0.71	<0.0001	0.89	0.77	1.02	0.099
Resection	0.49	0.48	0.51	<0.0001	0.81	0.71	0.92	0.001
Radiotherapy								
None/Unknown	Reference				Reference			
Beam Radiation	0.54	0.52	0.56	<0.0001	1.12	0.97	1.29	0.120
Chemotherapy								
No	Reference				Reference			
Yes	0.61	0.59	0.63	<0.0001	1.08	0.97	1.2	0.180

CI – confidence interval

Table 3.2 Multivariate Analysis of known prognostic factors in long-term glioblastoma survivors diagnosed between the years 2010 – 2016.

	Cases from Diagnosis (N = 15,878)				Long-Term Survivors (N=2,155)			
	Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value
Year of Diagnosis	1	0.99	1.01	0.98	1.03	0.98	1.08	0.246
Age	1.03	1.03	1.03	<0.0001	1.02	1.01	1.02	<0.0001
Surgery								
No Surgery	Reference				Reference			
Biopsy	0.65	0.62	0.68	<0.0001	0.84	0.67	1.04	0.105
Subtotal								
Resection	0.63	0.57	0.69	<0.0001	0.84	0.6	1.16	0.291
Total Resection	0.48	0.45	0.5	<0.0001	0.78	0.63	0.97	0.026
Radiotherapy								
None/Unknown	Reference				Reference			
Beam Radiation	0.55	0.52	0.58	<0.0001	0.9	0.68	1.19	0.460
Chemotherapy								
No	Reference				Reference			
Yes	0.53	0.5	0.56	<0.0001	1.27	0.97	1.66	0.079

CI – confidence interval

Table 3.3. Multivariate Analysis of known prognostic factors in long-term glioblastoma survivors diagnosed between the years 2010 – 2016 by age-group.

	20-44							
	All Cases (N=1331)				Long-Term Survivors (N=420)			
	Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value
Year of Diagnosis	1.03	0.98	1.07	0.262	1.04	0.92	1.18	0.485
Age	1.03	1.02	1.04	<0.0001	1.01	0.99	1.03	0.397
Surgery								
No Surgery	Reference				Reference			
Biopsy	0.71	0.58	0.88	0.002	1	0.62	1.63	0.999
Subtotal Resection	0.61	0.41	0.92	0.02	0.84	0.38	1.85	0.659
Total Resection	0.55	0.44	0.69	<.0001	0.77	0.47	1.28	0.32
Radiotherapy								
None/Unknown	Reference				Reference			
Beam Radiation	0.62	0.47	0.81	<.001	0.81	0.44	1.48	0.496
Chemotherapy								
No	Reference				Reference			
Yes	0.68	0.53	0.88	0.003	1.26	0.71	2.24	0.429

Table 3.3. Multivariate Analysis of known prognostic factors in long-term glioblastoma survivors diagnosed between the years 2010 – 2016 by age-group. (continued)

45-64								65+							
All Cases (N=7542)				Long-Term Survivors (N=1,254)				All Cases (N=7005)				Long-Term Survivors (N=481)			
Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value	Hazard Ratio	Lower 95CI	Upper 95CI	P Value
1.01	0.99	1.03	0.201	1.02	0.97	1.09	0.425	0.98	0.97	1	0.033	1.03	0.94	1.13	0.579
1.03	1.02	1.03	<0.0001	1.01	1	1.02	0.078	1.03	1.03	1.03	<0.0001	1.02	1	1.04	0.049
Reference				Reference				Reference				Reference			
0.63	0.59	0.69	<0.0001	0.78	0.59	1.04	0.088	0.66	0.62	0.71	<0.0001	0.85	0.53	1.38	0.512
0.61	0.53	0.7	<0.0001	0.86	0.57	1.3	0.477	0.65	0.56	0.75	<0.0001	0.71	0.32	1.55	0.383
0.47	0.43	0.51	<0.0001	0.79	0.6	1.05	0.102	0.48	0.45	0.52	<0.0001	0.73	0.45	1.19	0.21
Reference				Reference				Reference				Reference			
0.58	0.53	0.64	<0.0001	0.9	0.58	1.39	0.63	0.52	0.48	0.56	<0.0001	0.95	0.57	1.56	0.830
Reference				Reference				Reference				Reference			
0.55	0.5	0.6	<0.0001	1.36	0.9	2.05	0.146	0.51	0.47	0.55	<0.0001	1.13	0.72	1.78	0.595

CI – confidence interval

Supplementary Tables

Supplementary Table 3.1. Trends in glioblastoma incidence rates by age-group, 2001 - 2016

Age	Trend 1		Trend 2	
	Years	APC (95% CI)	Years	APC (95% CI)
20-29	2001-2016	1 (-0.3, 2.4)		
30-39	2001-2016	1.2 (0.3, 2.0) **		
40-49	2001-2016	-0.4 (-1.0, 0.1)		
50-59	2001-2016	0 (-0.3, 0.3)		
60-69	2001-2011	0.6 (0.1, 1.1) **	2011-2016	-0.8 (-2.0, 0.3)
70-79	2001-2008	1.4 (0.4, 2.4) **	2008-2016	-0.1 (-0.9, 0.6)
80+	2001-2016	1.1 (0.6, 1.7) **		

**P<0.05

APC – Annual Percentage Change

CI – Confidence Interval

Supplementary Table 3.2. Glioblastoma Age-adjusted incidence rates, 2001 - 2016

Year of Diagnosis	Overall			Males			Females			Incidence Rate (Male:Female)
	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	
2001	7,776	4.16	(4.07-4.26)	4490	5.05	(4.90-5.20)	3286	3.34	(3.22-3.47)	1.51
2002	8,051	4.22	(4.13-4.32)	4701	5.18	(5.03-5.33)	3350	3.36	(3.25-3.48)	1.54
2003	8,320	4.25	(4.16-4.35)	4804	5.13	(4.98-5.27)	3516	3.42	(3.31-3.54)	1.50
2004	8,724	4.34	(4.25-4.43)	5064	5.32	(5.17-5.47)	3660	3.50	(3.39-3.62)	1.52
2005	8,928	4.38	(4.29-4.47)	5156	5.26	(5.12-5.41)	3772	3.58	(3.46-3.69)	1.47
2006	9,058	4.35	(4.26-4.44)	5159	5.16	(5.01-5.30)	3899	3.62	(3.51-3.74)	1.42
2007	9,380	4.41	(4.32-4.50)	5439	5.32	(5.18-5.47)	3941	3.59	(3.48-3.71)	1.48
2008	9,638	4.45	(4.36-4.54)	5613	5.37	(5.23-5.51)	4025	3.58	(3.47-3.70)	1.50
2009	9,662	4.36	(4.27-4.44)	5584	5.21	(5.07-5.35)	4078	3.57	(3.46-3.68)	1.46
2010	9,958	4.40	(4.31-4.49)	5754	5.24	(5.11-5.38)	4204	3.59	(3.48-3.70)	1.46
2011	10,131	4.39	(4.31-4.48)	5931	5.28	(5.14-5.41)	4200	3.53	(3.42-3.63)	1.50
2012	10,584	4.47	(4.39-4.56)	6126	5.32	(5.19-5.46)	4458	3.66	(3.56-3.77)	1.45
2013	10,724	4.43	(4.35-4.52)	6222	5.30	(5.17-5.44)	4502	3.63	(3.53-3.74)	1.46
2014	10,765	4.35	(4.27-4.43)	6376	5.30	(5.17-5.43)	4389	3.44	(3.34-3.54)	1.54
2015	11,136	4.43	(4.35-4.51)	6448	5.26	(5.13-5.39)	4688	3.63	(3.52-3.73)	1.45
2016	11,332	4.40	(4.31-4.48)	6625	5.27	(5.15-5.40)	4707	3.56	(3.45-3.66)	1.48

AAIR – Age-adjusted incidence rate

CI – Confidence Interval

Supplementary Table 3.3 Glioblastoma Age-adjusted incidence rates by age-group, 2001 - 2016

Year of Diagnosis	20-29			30-39			40-49			50-59			60-69			70-79			80+		
	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)	Count	AAIR	(95% CI)
2001	93	0.25	(0.20-0.30)	297	0.70	(0.62-0.78)	926	2.23	(2.08-2.37)	1728	5.43	(5.18-5.69)	2021	9.76	(9.34-10.20)	1988	9.14	(8.61-9.70)	723	7.56	(7.02-8.13)
2002	140	0.35	(0.29-0.41)	302	0.70	(0.62-0.78)	911	2.12	(1.98-2.26)	1827	5.48	(5.23-5.74)	2102	9.99	(9.57-10.43)	2058	9.53	(8.98-10.09)	711	7.26	(6.73-7.81)
2003	129	0.35	(0.29-0.41)	286	0.65	(0.58-0.73)	1004	2.33	(2.18-2.47)	1926	5.58	(5.34-5.84)	2225	10.08	(9.66-10.51)	2033	9.33	(8.78-9.88)	717	7.06	(6.55-7.60)
2004	131	0.30	(0.25-0.35)	302	0.70	(0.62-0.78)	972	2.18	(2.04-2.32)	2053	5.68	(5.44-5.94)	2306	10.08	(9.67-10.50)	2141	9.90	(9.35-10.48)	819	7.87	(7.34-8.43)
2005	131	0.30	(0.25-0.35)	309	0.75	(0.67-0.84)	951	2.13	(1.99-2.27)	2145	5.73	(5.49-5.98)	2355	10.04	(9.63-10.45)	2159	10.01	(9.45-10.60)	878	8.36	(7.81-8.93)
2006	131	0.30	(0.25-0.35)	276	0.65	(0.58-0.73)	995	2.23	(2.09-2.37)	2212	5.67	(5.43-5.91)	2462	10.21	(9.81-10.62)	2084	9.63	(9.07-10.19)	898	8.36	(7.82-8.93)
2007	156	0.35	(0.29-0.41)	292	0.70	(0.62-0.79)	1005	2.27	(2.13-2.42)	2213	5.58	(5.35-5.81)	2672	10.39	(10.00-10.79)	2206	10.16	(9.60-10.75)	836	7.67	(7.15-8.20)
2008	140	0.35	(0.29-0.41)	286	0.70	(0.62-0.79)	1001	2.28	(2.14-2.42)	2253	5.58	(5.36-5.82)	2752	10.24	(9.86-10.62)	2269	10.36	(9.79-10.94)	937	8.47	(7.93-9.03)
2009	136	0.30	(0.25-0.35)	257	0.60	(0.53-0.68)	929	2.13	(1.99-2.27)	2194	5.32	(5.10-5.55)	2897	10.31	(9.93-10.69)	2224	10.05	(9.49-10.62)	1025	9.18	(8.62-9.75)
2010	146	0.35	(0.29-0.41)	275	0.70	(0.62-0.79)	993	2.28	(2.14-2.43)	2326	5.52	(5.30-5.75)	3025	10.24	(9.87-10.60)	2288	10.22	(9.66-10.80)	905	8.02	(7.50-8.55)
2011	150	0.35	(0.29-0.41)	311	0.80	(0.71-0.89)	953	2.23	(2.08-2.37)	2240	5.23	(5.02-5.45)	3279	10.70	(10.34-11.07)	2244	9.81	(9.27-10.37)	954	8.37	(7.85-8.92)
2012	172	0.40	(0.34-0.46)	311	0.75	(0.67-0.84)	848	2.03	(1.89-2.17)	2386	5.47	(5.25-5.70)	3384	10.61	(10.25-10.97)	2478	10.52	(9.97-11.09)	1005	8.72	(8.19-9.28)
2013	130	0.30	(0.25-0.35)	285	0.70	(0.62-0.79)	908	2.18	(2.04-2.32)	2526	5.72	(5.50-5.95)	3363	10.19	(9.85-10.54)	2507	10.21	(9.68-10.76)	1005	8.67	(8.14-9.22)
2014	195	0.40	(0.34-0.46)	335	0.80	(0.72-0.89)	835	2.02	(1.89-2.16)	2428	5.49	(5.27-5.71)	3420	10.02	(9.68-10.36)	2545	9.95	(9.44-10.49)	1007	8.68	(8.14-9.23)
2015	139	0.30	(0.25-0.35)	347	0.85	(0.76-0.94)	902	2.23	(2.08-2.37)	2572	5.77	(5.55-6.00)	3491	9.86	(9.54-10.20)	2643	10.04	(9.52-10.56)	1042	8.88	(8.35-9.44)
2016	165	0.35	(0.30-0.41)	335	0.80	(0.72-0.89)	801	1.97	(1.83-2.11)	2434	5.48	(5.27-5.71)	3809	10.38	(10.05-10.71)	2766	10.21	(9.71-10.74)	1022	8.58	(8.06-9.12)

AAIR – Age-adjusted incidence rate

CI – Confidence Interval

Supplementary Table 3.4. Patient characteristics of glioblastoma patients diagnosed between 2000 – 2016.

	All Cases		Long-Term Survivors		
	N	Percent	N	Percent	
Total	35,236	100.00%	5,026	100.00%	
Age Groups					
20-44	3,330	9.45%	1,161	23.10%	P<0.0001
45-64	16,729	47.48%	2,890	57.50%	
65-85+	14,422	43.07%	975	19.40%	
Sex					
Male	20,616	58.51%	2,922	58.14%	P=0.5646
Female	14,620	41.49%	2,104	41.86%	
Race					
American Indian/Alaska Native	134	0.38%	23	0.46%	P<0.0001
Asian or Pacific Islander	1,588	4.51%	304	6.05%	
Black	2,012	5.71%	311	6.19%	
Unknown	72	0.20%	13	0.26%	
White	31,430	89.20%	4,375	87.05%	
First Line Therapy					
Surgery					
Biopsy	11,200	31.79%	1,607	31.97%	P<0.0001
Subtotal Resection	5,598	15.89%	723	14.39%	
Total Resection	11,245	31.91%	2,244	44.65%	
Other	448	1.27%	83	1.65%	
None	6,745	19.14%	369	7.34%	
Radiotherapy					
External Beam	25,748	73.07%	4,508	89.69%	P<0.0001
None/Unknown	8,967	25.45%	429	8.54%	
Other	521	1.48%	89	1.77%	
Chemotherapy					
Yes	21,664	61.48%	4,270	84.96%	P<0.0001
No/Unknown	13,572	38.52%	756	15.04%	

Supplementary Table 3.5. Age-Standardised Relative Survival Rates, 2000 – 2016.

Year	N	Age-Standardised Relative Survival						
		Median	1 year	95% CI	2 year	95% CI	5 year	95% CI
2000-2016	35,236	11	45.30%	(44.7-45.8)	19.50%	(19.1-20)	5.80%	(5.5-6.1)
2000	1,737	8	33.60%	(31.3-35.8)	11.50%	(10-13.2)	4.20%	(3.2-5.3)
2001	1,717	9	34.40%	(32.2-36.7)	10.20%	(8.8-11.9)	3.30%	(2.5-4.4)
2002	1,775	9	36.60%	(34.4-38.9)	12.80%	(11.2-14.5)	3.80%	(2.9-4.9)
2003	1,865	9	39.00%	(36.8-41.2)	14.40%	(12.8-16.1)	4.70%	(3.8-5.9)
2004	1,978	10	41.40%	(39.3-43.6)	17.80%	(16-19.6)	6.10%	(5-7.4)
2005	1,909	11	42.20%	(40-44.3)	19.50%	(17.7-21.4)	6.10%	(5-7.4)
2006	1,890	10	44.50%	(42.3-46.6)	20.10%	(18.3-22)	6.40%	(5.3-7.7)
2007	2,043	11	45.80%	(43.6-47.9)	20.20%	(18.5-22.1)	5.80%	(4.8-7)
2008	2,083	11	46.20%	(44.1-48.3)	20.10%	(18.3-21.9)	5.50%	(4.5-6.6)
2009	2,065	12	48.50%	(46.4-50.7)	22.00%	(20.1-23.9)	6.70%	(5.5-8)
2010	2,124	13	50.40%	(48.3-52.5)	23.00%	(21.1-24.9)	6.00%	(4.9-7.3)
2011	2,192	12	48.80%	(46.7-50.8)	22.80%	(21-24.7)	6.70%	(5.5-7.9)
2012	2,343	12	49.60%	(47.5-51.6)	23.50%	(21.7-25.4)		
2013	2,326	13	51.40%	(49.4-53.4)	23.90%	(22.1-25.8)		
2014	2,339	12	49.90%	(47.8-51.9)	21.50%	(19.8-23.4)		
2015	2,413	12	49.50%	(47.4-51.5)				

CI- Confidence Intervals

Supplementary Table 3.5 Age-Standardised Relative Survival Rates, 2000 – 2016. (continued)

Age-Standardised 1 year Conditional Survival							
12 months	95% CI	24 months	95% CI	36 months	95% CI	48 months	95% CI
40.70%	(39.8-41.6)	54.60%	(53-56.2)	67.00%	(64.6-69.3)	72.70%	(69.4-75.6)
28.90%	(24.8-33)	49.90%	(37-61.4)	76.40%	(64.8-84.6)	81.80%	(56.4-93.2)
25.20%	(21.6-28.9)	50.30%	(37.5-61.8)	63.00%	(41.8-78.3)	83.30%	(68.4-91.6)
30.10%	(26.4-33.9)	42.10%	(36.3-47.8)	-	-	-	-
35.10%	(31.3-38.8)	55.80%	(48.5-62.5)	68.90%	(58.7-77.1)	76.10%	(62.6-85.2)
38.70%	(35.1-42.3)	61.50%	(54.7-67.6)	71.00%	(61.6-78.5)	77.20%	(67-84.6)
44.50%	(40.6-48.4)	55.20%	(49.1-60.9)	71.50%	(61.4-79.3)	71.10%	(60.1-79.7)
42.10%	(38.4-45.8)	55.40%	(48.8-61.6)	69.70%	(60.6-77.1)	68.60%	(57.4-77.5)
42.60%	(39.1-46.1)	55.10%	(49.2-60.6)	64.00%	(55.6-71.1)	77.90%	(68.6-84.7)
42.40%	(38.8-45.9)	56.80%	(51-62.2)	63.80%	(55.9-70.6)	74.90%	(65-82.5)
43.30%	(39.9-46.7)	56.50%	(50.8-61.8)	62.60%	(54.7-69.5)	71.50%	(61.2-79.5)
43.30%	(40-46.5)	52.30%	(47-57.4)	63.90%	(55.6-71)	67.70%	(57.2-76.1)
44.00%	(40.7-47.2)	59.00%	(53.3-64.2)	65.50%	(57.6-72.3)	67.70%	(57-76.3)
44.80%	(41.6-47.9)	53.10%	(47.7-58.1)	70.30%	(62.5-76.7)		
44.30%	(41.2-47.4)	53.10%	(48-58)				
40.70%	(37.7-43.8)						

Supplementary Table 3.6. Patient characteristics for glioblastoma patients diagnosed between 2010 – 2016.

	Glioblastoma Cases diagnosed 2010-2016	
	N	Percent
Total	16,174	100.00%
Age Groups		
20-44	1361	8.41%
45-64	7697	47.59%
65-85+	7116	44.00%
Sex		
Male	9446	58.40%
Female	6728	41.60%
Race		
American Indian/Alaska Native	71	0.44%
Asian or Pacific Islander	868	5.37%
Black	955	5.90%
Unknown	54	0.33%
White	14226	87.96%
First Line Therapy		
Surgery		
Biopsy	7315	45.23%
Subtotal Resection	583	3.60%
Total Resection	5453	33.71%
Other	154	0.95%
None	2669	16.50%
Radiotherapy		
External Beam	12183	75.32%
None/Unknown	3844	23.77%
Other	147	0.91%
Chemotherapy		
Yes	11508	71.15%
No/Unknown	4666	28.85%

Supplementary Table 3.7. Patient characteristics for glioblastoma patients diagnosed between 2010 – 2016 by treatment status.

	Complete Standard of Care		No/Unknown Treatment		
	N	Percent	N	Percent	
Total	9555	100.00%	946	100.00%	
Age Groups					
20-44	952	9.96%	41	4.33%	P<0.001
45-64	5154	53.94%	274	28.96%	
65-84	3449	36.10%	631	66.70%	
Sex					
Male	5658	59.22%	517	54.65%	P<0.01
Female	3897	40.78%	429	45.35%	
Race					
American Indian/Alaska Native	45	0.47%	1	0.11%	P=0.038
Asian or Pacific Islander	486	5.09%	56	5.92%	
Black	529	5.54%	51	5.39%	
Unknown	24	0.25%	4	0.42%	
White	8471	88.66%	834	88.16%	

Supplementary Table 3.8. Patient characteristics for glioblastoma patients diagnosed between 2010 – 2016 by age-group.

GBM Cases Diagnosed 2010-2016	20-44 years		45-64 years		65+ years	
	N	Percent	N	Percent	N	Percent
Total	1361	100.00%	7697	100.00%	7116	100.00%
Sex						
Male	856	62.89%	4691	60.95%	3899	54.79%
Female	505	37.11%	3006	39.05%	3217	45.21%
Race						
American Indian/Alaska Native	9	0.66%	39	0.51%	23	0.32%
Asian or Pacific Islander	139	10.21%	380	4.94%	349	4.90%
Black	107	7.86%	529	6.87%	319	4.48%
Unknown	12	0.88%	22	0.29%	20	0.28%
White	1094	80.38%	6727	87.40%	6405	90.01%
First Line Therapy						
Surgery						
Biopsy	651	47.83%	3537	45.95%	3127	43.94%
Subtotal Resection	45	3.31%	305	3.96%	233	3.27%
Total Resection	497	36.52%	2741	35.61%	2215	31.13%
Other	14	1.03%	76	0.99%	64	0.90%
None	154	11.32%	1038	13.49%	1477	20.76%
Radiotherapy						
External Beam	1125	82.66%	6252	81.23%	4806	67.54%
None/Unknown	220	16.16%	1361	17.68%	2263	31.80%
Other	16	1.18%	84	1.09%	47	0.66%
Chemotherapy						
Yes	1104	81.12%	6057	78.69%	4347	61.09%
No/Unknown	257	18.88%	1640	21.31%	2769	38.91%

Chapter 4: A new systemic disease mouse model for glioblastoma capable of single tumour cell detection

Key Points

Questions Where do tumour cells go beyond the tumour mass and where do recurrent tumour come from?

Findings An orthotopic glioblastoma xenograft mouse model using a highly sensitive luciferase system detected tumour cells in all areas of mouse brain sampled and in some cases as single cells, irrespective of whether invasion was observed at the primary tumour. Analysis of blood and lung tissue also detected the presence of tumour cells providing a viable mechanism of haematogenous dissemination.

Conclusion Glioblastoma is a systemic brain disease with the presence of residual tumour cells ubiquitously disseminated throughout the brain

4.1 Abstract

Importance Glioblastoma is characterised by extensive infiltration into the brain parenchyma leading to inevitable tumour recurrence and therapeutic failure. Future treatments will need to target the specific biology of tumour recurrence, but our current understanding of the underlying mechanisms is limited. Significantly there is a lack of available methods and models that are tailored for the examination of tumour recurrence.

Objective To develop a highly sensitive glioblastoma mouse model for the investigation of recurrence biology and therapeutic efficacy.

Design, Setting, and Participants In this study, NOD-SCID mice were orthotopically implanted with luciferase-labelled donor U87MG or MU20 glioblastoma cells. Four days later an unlabelled recipient tumour was implanted on the contralateral side. Mice were euthanised at humane-end point and tissue and blood samples were collected for *ex vivo* analysis.

Main Outcomes and Measures Mice brains were cryosectioned and stained with haematoxylin and eosin to identify the primary labelled tumour and invasive margin. Blood and explanted tissue samples were lysed and tested for luciferase activity and tumour cell numbers measured using predetermined standard curves.

Results *Ex vivo* analysis of firefly-labelled MU20 tumours displayed extensive invasion at the primary tumour margin, whereas firefly-labelled U87MG tumours exhibited an expansive phenotype with no evident invasion at the tumour margin. Luciferase signals were detected in the contralateral unlabelled recipient tumours for both U87MG and MU20 tumours compared to non-implanted control brain (U87MG $P=.01$; MU20 $P=.009$). Remarkably, tumour cells were uniformly detected in all tissue samples of the supratentorial brain region compared to control tissue (U87MG $P=.02$; MU20 $P=.04$), with single tumour cells detected in some tissue samples (mean [range], U87MG: 1.3 cells/5mg [0.2 – 4.2 cell/5mg]; MU20: 695.2 cells/5mg [33.9 – 2399 cells/5mg]). Circulating tumour cells were also detected in jugular blood of most xenograft mice (mean [range], U87MG: 3.5 cells/5 μ L [1.0 – 6.6 cells/5 μ L]; MU20: 6.8 cells/5 μ L [1.0 – 16.6 cells/5 μ L]; detected from 3/4 mice for each group). Moreover, tumour cells were detected in the lungs of all mice ($P<.001$), a probable event of haematogenous dissemination. Similar results were obtained when the U87MG cells were labelled differently with gaussian luciferase.

Conclusions and Relevance These findings describe a systemic disease model for glioblastoma, which can be used to investigate recurrence biology and therapeutic efficacy towards recurrence.

4.2 Introduction

Glioblastoma is the most common malignant brain and CNS tumour with an incidence of 3.21 per 100,000 population in the US.⁷ Patients currently receive an aggressive standard of care consisting of maximal surgical resection, radiotherapy and chemotherapy with temozolomide, yet overall survival remains dismal at approximately 15 months.³⁸⁷ Tumour recurrence is the major cause of death, most likely originating from tumour infiltration that escapes surgical resection. Recent clinical trials have focused on assessing novel therapies against glioblastoma recurrence using a wide range of drug classes and targets, yet almost 2000 clinical trials (including over 1000 trials in Phase II or above) have failed to provide any meaningful improvement to survival and few lead to FDA approval.³⁸⁸ This is in contrast to the reported phase II and III clinical success rates of 23% and 45% observed for oncology-related drugs representing a misaligned drug development process.²¹⁰ The current inability to predict clinical response highlights a discordance in the current approaches to clinical trial development for glioblastoma and requires a re-evaluation of therapeutic development strategies.

Epithelial Growth Factor Receptor (EGFR) has been a significant target for therapeutic development in Glioblastoma owing to the high prevalence of *EGFR* amplification and its important role as a key survival signalling pathway.^{21,389,390} Initial drug development focussing on monoclonal antibodies (Cetuximab) and Tyrosine Kinase Inhibitors (TKIs, Erlotinib and Gefinitib) demonstrated therapeutic efficacy in preclinical mice models measuring the growth rate of the primary tumour in subcutaneous and orthotopic intracranial glioblastoma implants.³⁹¹⁻³⁹⁷ Treatment with EGFR inhibitors also improved mice survival in orthotopic xenografts models further supporting its development into clinical trials. Despite these encouraging preclinical results, however, clinical trials have failed to recapitulate these results with no reported benefit to overall survival in newly diagnosed or recurrent glioblastoma.^{222,398-}

⁴⁰³ The use of EGFR inhibitors for concomitant or adjuvant therapy with the current standard of care has largely been abandoned as a clinical strategy due to these disappointing results.

Cilengitide, an $\alpha V\beta 3$ and $\alpha V\beta 5$ inhibitor, was developed for its potential targeting of angiogenesis and invasion, two hallmark characteristics of glioblastoma pathology. Cilengitide testing in preclinical orthotopic xenograft mice models demonstrated outstanding therapeutic efficacy, preventing primary tumour growth beyond 1-2mm for the duration of the experimental timeline (8-9 weeks).⁴⁰⁴ Phase II clinical trials, however, reported a lack of clinical efficacy in both newly diagnosed and recurrent glioblastoma.^{188,405} This was further confirmed in a phase III clinical trial of MGMT methylated patients where Cilengitide failed to provide a benefit to overall survival causing the manufacture to halt development of the drug as an anti-cancer treatment.¹⁹¹

Bevacizumab, a monoclonal anti-VEGF inhibitor, was once thought to be the most promising drug candidate since temozolomide and remains the most extensively clinical tested drug for glioblastoma. Early preclinical studies confirmed specific inhibition of angiogenesis and tumour growth of primary orthotopic tumour implants, leading to an improvement in survival in mice models.^{406,407} The translation to clinical trials, however, has not been successful providing no improvement to overall survival in newly diagnosed and recurrent glioblastoma.^{99,100,170,176,178,197} Further preclinical studies looking into the failure of Bevacizumab treatment have reported that it may enhance tumour infiltration leading to poor patient outcome,⁴⁰⁸ and suggest that primary tumour response may not be a predictor for clinical response.

The poor predictive value of preclinical studies testing therapeutic drugs has been observed in almost all clinical trials since the approval of temozolomide, questioning the validity of our preclinical measurements and whether they truly represent the clinical target. Current

preclinical models used to predict therapeutic efficacy most often report the response of the primary tumour, clinically however the primary tumour is removed, and tumour response is commonly reported as response rate or progression-free-survival, which is a measure of recurrent tumour progression.¹⁷³ The resulting patient overall survival and cause of death is therefore attributed to the recurrent tumour, which accumulating evidence suggests is biologically dissimilar to the primary tumour.²²⁰ It is therefore expected that treatments that are effective against the primary tumour may not show the same efficacy in the recurrent setting explaining the poor translation to clinical trials. The advancement of new therapies for glioblastoma therefore requires a deeper knowledge of the molecular mechanisms and novel targets of tumour recurrence. Advancements in this area, however, have been limited in humans due to the difficulty in obtaining quality tissue specimens and an inability to sensitively track clinical progression and identify the origin of tumour recurrence.²²¹ Animal models are necessary to overcome these limitations and further develop our understanding of recurrence biology. Currently, however, there are no standard mouse models available to study tumour recurrence with most models focussing on the primary tumour. While some models have used tumour infiltration quantitation to investigate the molecular mechanisms of tumour progression, these approaches fail to capture tumour infiltration throughout the entire brain limiting inferences regarding tumour recurrence origin.⁴⁰⁹⁻⁴¹²

Recent advances in tumour cell detection technology have utilised luciferase-based tumour cell labelling for *in vivo* detection.⁴¹³⁻⁴¹⁷ The substrate luciferin is oxidised in the presence of a luciferase enzyme producing a luminescence signal that is highly specific and sensitive. The luciferase system is highly reproducible and offers the additional benefit of quantitative analysis of tumour cells. While this system has been utilised for the detection of CTCs and tumour cell monitoring *in vivo* previously, single tumour cell detection has remained elusive.⁴¹⁸ Additionally, limitations in current methods often require centrifugation and cell enrichment

processes, which result in substantial loss of tumour cells and inaccurate quantification of true tumour cell number. Using luciferase technology, we have developed a systemic brain disease model of glioblastoma capable of quantitating tumour presence in mouse tissue with single cell sensitivity.

4.3 Materials and Methods

Cell culture

The human glioblastoma cell line U87MG was obtained from the American Type Culture Collection (ATCC, USA). Human glioblastoma cell line MU20 was derived from a patient at the Royal Melbourne Hospital (Parkville). Cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, USA) containing 10% foetal bovine serum (Thermo Fisher Scientific, USA), 2mM glutamine, 100U/ml penicillin and 100µl/ml streptomycin (Invitrogen, USA). Cells were incubated in a humidified atmosphere of 10% CO₂ 37°C.

Stable transfection of firefly and gaussia luciferase

U87MG cells were stably transfected with pGL4.51[luc2/CMV/Neo] (Promega, USA) or pCMV-Gluc-KDEL (Prolume Ltd., USA) vector using Fugene® HD (Promega). Cells underwent selection with 2mg/ml G418 (Roche, Basel, Switzerland) and stable clonal colonies obtained.

Animal experiments

Animal experimentation was approved by the University of Melbourne Animal Ethics Committee. 6-8 week female NOD-SCID mice (Animal Resource Centre, WA, Australia). For intracranial implantation, mice were anaesthetized with 4% isoflurane and maintained at 2% isoflurane for the procedure. Mice were shaved and positioned on the stereotactic. A small burr hole <1mm diameter was fashioned and 5×10^4 or 2.5×10^5 firefly-labelled cells implanted in 5µL using a 27-gauge needle at a flow rate of 1µL/min. The needle was retracted, and the hole sealed using sterile bone wax. Mice were monitored for recovery and 4 days later a non-labelled contralateral tumour was implanted using the same procedure. Following experimental end-

point, mice are euthanised and organs snap-frozen. For cerebral blood collection, the jugular vein is ligated above the clavicle and the vein incised.

Ex vivo luciferase analysis

Frozen tissue was cut into ~25mg pieces and homogenised in cell culture lysis reagent (Promega, USA). Tissue lysates were placed at 4°C for 30mins with agitation and transferred to an opaque 96 well plate for luminescence reading using a luciferase assay kit (Promega) and luminometer (Promega). Luciferase activity is presented as relative luciferase units (R.L.U).

Statistical analysis

All statistical analyses were performed in Graphpad Prism (GraphPad Software Inc, USA). Single cell quantitation was determined using best-fit linear regression analysis and cell number extrapolated for positive luciferase samples. An unpaired student's t test was used to compare between groups.

4.4 Results

While glioblastoma is known to be a highly invasive brain tumour, most studies fail to consider the consequences of tumour infiltration when developing new treatments in a preclinical setting. Yet the importance of addressing tumour infiltration for long-term patient survival has been well established since the introduction of a surgical standard for glioblastoma. Walter Dandy in 1928, famously demonstrated that radical hemispherectomy of the diseased brain was incurable with tumour recurrence developing in the contralateral hemisphere.²²² These early experiments provided evidence of widespread dissemination of glioblastoma throughout the brain that is not captured in current animal models. Specifically, limitations in tumour detection technology prevent close examination of the inconspicuous locations of the brain where residual tumour cells may reside and form eventual recurrence. We therefore sought to develop a model capable of accurately detecting all tumour cells present within any section of the mouse brain. To achieve the required sensitivity we used luciferase labelling, which can provide a reproducible luminescence signal with minimal background that is capable of single cell detection.

We stably transfected U87MG glioblastoma tumour cells with pGL4.51[luc2/CMV/Neo] as described in the methods. After selecting the clonal population with the highest luciferase signal per cell, we set out to confirm the sensitivity of our U87MG-F-Luc labelled cells for single tumour cell detection in mouse tissue. First, we analysed the sensitivity of our labelled cells in brain tissue by performing a titration down to single cells. Regression analysis reported a strong linear relationship ($R^2=0.99$) with minimal variation in luciferase signal. Single tumour cells produced a signal of 464 Relative Luciferase Units (R.L.U) per cell in 5mg brain tissue, clearly identifiable above the background signal of 19 R.L.U for control brain tissue (Figure 1A) and providing sensitive and specific detection of single tumour cells in mouse brain.

Recently, the presence of circulating tumour cells (CTCs) in blood has been widely accepted as an inherent biology of glioblastoma, though reliable detection of CTCs remains difficult.⁴¹⁹⁻
⁴²¹ While the tumourigenic capacity of CTCs has been evident with the development of extracranial metastasis, its relevance in tumour progression within the brain remains unclear.⁴²² Recently it was reported that glioblastoma CTCs self-seed to the primary tumour *in vivo* establishing a potential mechanism of hematogenous dissemination within the brain.⁴²³ We therefore decided to test blood and lung tissue for evidence of CTCs using our glioblastoma mouse model. Performing a titration down to single cells using U87MG-F-Luc tumour cells determined a single cell signal of 58 R.L.U in 5 μ L blood and 362 R.L.U in 5mg lung tissue ($R^2=0.99$) (Figure 1B-C). Additionally, detection of luciferase activity in the absence of tumour cells provided a background reading of approximately of 12 R.L.U for lung tissue and blood; a negligible signal compared to single tumour cell activity. These results therefore demonstrate a sensitive and reproducible method for single tumour cell detection in mouse tissue and blood. To test the application of our model using different glioblastoma cell lines we generated a MU20-F-Luc cell line and determined the single cell signal within brain, blood and lung (Supplementary Figure 1A-C).

Having confirmed sensitive single tumour cell detection *in vitro*, we next applied our technique to investigate tumour infiltration in a glioblastoma mouse model. There are currently few models available for specifically investigating glioblastoma infiltration that also recapitulates the most recent discoveries of glioblastoma biology.^{424,425} We therefore adapted a self-seeding breast cancer model utilising orthotopic contralateral tumour implantations for our study.⁴²⁶ U87MG or MU20 Firefly-Luciferase labelled donor Glioblastoma tumour cells were implanted into the basal ganglia of one hemisphere, while unlabelled recipient glioblastoma tumour cells were implanted in the contralateral hemisphere 4 days later (Figure 2A). Mice were monitored

until euthanasia following strict humane-end point criteria. Two mice brains from each group were snap-frozen in O.C.T compound for cryosectioning. The 4 remaining mice tumours were explanted, and peripheral brain samples collected from the frontal lobe and caudal-side of the parietal-temporal/occipital lobe distal to primary tumours (Figure 2B). Mice implanted with MU20 tumours had a shorter median survival time of 28 days compared to 36 days for U87MG implanted tumours ($P=.03$) (Figure 2C), displaying increased cellularity and invasive patterns (Figure 2D-G). MU20-F-Luc labelled tumours displayed an aggressive invasive margin with tumour clusters leading the invasive front. U87MG-F-Luc tumour cells in contrast, exhibited a contained expansive growth of the primary tumour with no identifiable invasive margin present.

To characterise the extent of tumour infiltration around the brain we first examined the luciferase activity in our labelled donor and unlabelled recipient U87MG tumour explants. We detected positive luciferase signals in the unlabelled recipient tumour explant compared to negative control non-implanted mouse brain tissue ($P=.01$) (Figure 3A). Furthermore, analysis of the peripheral brain tissue was positive for luciferase activity in comparison to non-implanted control mouse brain ($P=.02$) (Figure 3B). Luciferase activity was also detected in blood and lung samples of mice (blood, $P=.04$; lung tissue, $P<.001$). Intriguingly, while luciferase activity was detected in the lungs of all mice, luciferase activity was only detected in blood of 3 of the 4 mice (Supplementary Figure 2A). Luciferase signal was also detected in other mice organs sampled (Supplementary Figure 3A). Quantitation of U87MG-F-Luc tumour using the standard curve from Figure 1 determined a mean of 5325 cells/5mg brain tissue (range: 531 – 11,116 cells/5mg) in the firefly-labelled donor explant and 3.9 cells/5mg brain tissue (range: 0.8 – 10.3 cells/5mg) in the unlabelled recipient explant (Figure 1C). Tumour cell quantitation also demonstrated the presence, though lower tumour cell numbers in all peripheral brain samples (mean: 1.3 cells/5mg, range: 0.2 – 4.2 cells/5mg) and lung tissue (mean: 0.4 cells/5mg, range: 0.1 – 0.6 cells/5mg). Since not all mice were positive for luciferase

activity and euthanised at different times, only mice with positive blood samples were quantitated to determine the relative CTC number when present. In these jugular blood samples, a mean of 3.5 cells/5µL blood (range: 1.0 – 6.6 cells/5µL) was detected. These results characterise a systemic dissemination of glioblastoma cells across both hemispheres of the brain and in blood.

We next set out to characterise the infiltration of the MU20-F-Luc implanted mice. Similar to our results from U87MG-F-Luc implanted mice, luciferase activity was detected in the labelled donor and unlabelled recipient tumours as well as all peripheral brain and lung tissue samples collected (unlabelled recipient tumour, $P=.009$; peripheral brain, $P=.04$; lung tissue $P<.001$) (Figure 2A-B). CTCs were also detected in the blood of 3 of the 4 mice ($P=.03$). Interestingly, most mouse organ tissues tested were positive for luciferase signal (Supplementary Figure 3B). Quantitation of tumour cell numbers in the labelled donor tumour determined a mean of 1,372,968 cells/5mg brain tissue (range: 23,889 – 3,182,974 cells/5mg) and 3898 cells/5mg brain tissue (range: 244 – 8,121 cells/5mg) in the unlabelled recipient tumour. Tumour cells were also present in all peripheral brain samples (mean: 695.2 cells/5mg, range: 33.9 – 2399 cells/5mg) but had a comparatively reduced tumour presence in blood (mean: 6.8 cells/5µL, range: 1.0 – 16.6 cells/5µL; positive samples only) and lung tissue (mean: 81.0 cells/5mg, range: 9.9 – 144.4 cells/5mg).

To ensure that the observed phenotype was not the result of firefly luciferase labelling, we generated a non-secreted gaussia luciferase labelled U87MG cell line. Single cell sensitivity was confirmed *in vitro* as described before ($R^2=0.99$) (Supplementary Figure 1D-F). U87MG-G-Luc labelled donor cells were intracranially implanted into the basal ganglia of mice and unlabelled recipient U87MG cells implanted contralaterally 4 days later. Given our previous model presented with detection of U87MG tumour cells within the peripheral brain tissue and blood of mice as early as 13 days post-implantation, we euthanised all mice at 16 days to test

the consistency of our model. Consistent with previous results, all mice tissue samples were positive for luciferase activity (unlabelled recipient tumour, $P=.03$; peripheral brain, $P<.001$, lung tissue, $P=.04$) (Figure 5A-B) although we did not detect any signal within collected blood samples ($P=0.31$). Quantitative analysis of tumour cell number demonstrated an average (mean $\pm$ SEM) of 734.0 ± 248.6 cells/5mg brain tissue within the labelled donor explant and 8.1 ± 4.8 cells/5mg brain tissue in the unlabelled recipient explant. In the peripheral brain samples an average of 1.4 ± 0.15 cells/5mg brain tissue was detected, which was lower than the presence of tumour cells within the lung (7.5 ± 2.4 cells/5mg). These results further confirm our findings of a systemic infiltration of tumour cells within the brain and the likely presence of CTCs; though not detected in our U87MG-G-Luc implanted mice.

4.5 Discussion

Infiltration of the brain parenchyma is thought to be the cause of tumour recurrence and patient mortality in glioblastoma.^{222,427} The extensive dissemination throughout the brain evades primary surgical resection and remains incurable with the current standard of care.³⁸⁷ While recurrent clinical trials can test the specificity of drugs for recurrent tumour cells, there is often limited existing preclinical evidence for efficacy in recurrent tumours, which have resulted in expensive and long clinical trials that ultimately have not delivered.^{388,428} Better models are needed to predict treatment response in recurrent glioblastoma and importantly establish the mechanisms regulating tumour recurrence.

In this study we have developed a novel quantitative model of glioblastoma tumour infiltration. We report the presence of tumour cells moving away from the labelled donor tumour to the unlabelled recipient tumour in both U87MG and MU20 xenograft cell lines. Remarkably, detection of tumour cells within the unlabelled recipient tumour was observed after only 13 days in the U87MG xenograft model suggesting highly motile tumour dissemination beginning in the early-stages of tumour development. Labelled tumour cell numbers increased in the unlabelled recipient tumour over time in both xenograft models, possibly due to expansion of labelled cells in the recipient tumour and/or continual infiltration from the labelled donor tumour. Interestingly, tumour cell infiltration was detected extensively throughout the distal regions of the supratentorial brain. A similar finding has been reported by Sahm *et al.*, (2012) who described the presence of single secondary glioblastoma tumour cells in peripheral and inconspicuous regions of the human brain.⁴²⁹ The authors stated that single tumour cells were located in virtually most areas of the brain consistent with a systemic disease of the brain. The validity of the conclusion for primary glioblastoma, however, has been questioned by the use of IDH1 mutation as a marker for tumour cells since IDH1 mutations in glioblastoma confer to

a genetically distinct and infrequent subset of glioblastoma with predicted better survival outcome.^{22,23} Our current study has confirmed and expanded on the extensive pattern of infiltration across the supratentorial brain in primary glioblastoma further supporting the concept of a systemic brain disease.

These findings, however, are unexpected for the U87MG xenograft model, which are commonly criticised for not recapitulating the invasive tumour margin seen clinically in glioblastoma.⁴³⁰⁻⁴³² The U87MG xenograft in our study failed to demonstrate invasion at the primary tumour margin, yet still progressed to whole brain dissemination raising the question as to whether primary tumour invasion is a requirement for recurrence progression.^{431,433} Until recently most brain tumour researchers and clinicians believed haematogenous dissemination was not possible in glioblastoma due to the rare incidence of clinical metastases.^{434,435} With advances in CTC detection technology, blood-borne glioblastoma cells have been isolated and gradually accepted as a characteristic of glioblastoma pathology, though the clinical relevance and regulation of these CTCs remains unclear.^{420,423,436-438} Here, we have detected both viable CTCs and lung micrometastases in both U87MG and MU20 xenograft models, supporting a functional pathway for haematogenous dissemination. CTCs may offer a novel mechanism of tumour dissemination throughout the brain and also explain the extensive dissemination of the U87MG xenograft model, despite its limited invasive capabilities. It will be of interest to further elucidate the molecular mechanism of how these CTCs are generated in glioblastoma and its implications for tumour recurrence.

To advance future therapeutic strategies for glioblastoma it will be important to recognise the biological development of recurrence. There is, however, still much debate about the role of progressive genetic mutations in the formation of recurrence. Körber *et al.*, (2019) have provided the most comprehensive overview of recurrence development using deep whole-genome-sequencing on primary and recurrent human glioblastoma tumours. They reported that

relapsed tumours did not acquire stereotypical patterns of mutations in recurrence and progressed from existing oligoclonal populations from the primary tumour.³⁶ This finding suggests that glioblastoma develops early and has acquired its driver mutations by the time of diagnosis. Our novel mouse model extends on this finding, using the existing genetic mutations of our xenograft cell lines to investigate tumour progression. Expanding on our findings, we propose that glioblastoma recurrence is preordained at the time of diagnosis whereby glioblastoma tumour cells have already infiltrated systemically throughout the brain. It is possible that this extensive infiltration of the brain may lead to residual cells that given time are capable of forming tumour recurrence, as witnessed in Walter Dandy's radical surgical experiments.²²² This provides significant implications for future treatment directions and presents the possibility of perpetual recurrence should residual tumour cells remain following treatment. Future research directions will require a comprehensive understanding of the distinct recurrent cell entities that exist at the time of diagnosis, which challenges our current focus on primary tumour biology.

The ability to measure tumour infiltration *in vivo* with use of this model will provide a significant advancement over existing preclinical models that have failed to predict therapeutic efficacy in the clinic. Our standardised mouse model allows for highly sensitive comparative analysis of tumour infiltration, representing the major failure for current therapeutic development and presenting with distinct biological properties to the primary tumour.

In summary, our single tumour cell detection method has uncovered novel characteristics of glioblastoma progression and recurrence that emphasises glioblastoma as a systemic disease of the brain. This model improves on previous methods of tumour cell detection and quantification, which have been limited by sensitivity or specificity.^{413-418,439-441} Our method overcomes these limitations, providing highly sensitive single tumour cell quantitation capable of detecting all viable tumour cells with no cell loss. The sensitive nature of our luciferase

system is ideal for both short- and long-term studies of tumour cell dissemination. Additionally, due to the small blood volume required for analysis, longitudinal monitoring of tumour cells can be performed to investigate the temporal relationship between early tumour cell dissemination and recurrence. This method, however, is restricted to animal models and cannot be used in a clinical setting. Despite this limitation, our method can provide important preclinical information regarding mechanisms of glioblastoma tumour recurrence under controlled conditions.

4.6 Conclusions

This novel mouse model of tumour infiltration can be used to advance our understanding of glioblastoma progression and provide new strategic research directions for development of new predictive biomarkers and therapies against recurrence.

Figure Legends

Figure 4.1. Single U87MG-F-Luc tumour cell quantitation *in vitro*.

U87MG-F-Luc cells were serially diluted to single cells and spiked with 5mg brain tissue (A), 5mg lung tissue (B) or 5µL blood (C). Cells were lysed and relative luciferase activity (R.L.U) measured (Left panels). Single cell quantitation was determined by best-fit linear regression analysis and was calculated at approximately 464 R.L.U, 362 R.L.U and 58 R.L.U in brain, lung and blood respectively (Right panels [Red box zoom]). Luciferase background was 19 R.L.U for brain tissue and 12 R.L.U for lung tissue and blood. Data is mean $\pm$ SEM, n=7 for brain and lung tissue and n=12 blood.

Figure 4.2. Orthotopic contralateral mouse implantation of U87MG and MU20 GBM cells display similar survival with different patterns of tumour invasion.

A, Contralateral tumours were implanted on day 1 and day 5 using 5×10^4 labelled or unlabelled tumour cells. Mice were monitored and euthanised at humane-end point. B, Brains were harvested for collection of peripheral brain tissue samples [Left Panel] and contralateral tumours [Right Panel]. C, Kaplan-Meier curve of overall survival for mice bearing MU20 and U87MG contralateral implantations. D-E, Microscopic images of immunohistochemistry analysis of labelled tumours for U87MG-F-Luc and MU20-F-Luc (F-G) at 100X original magnification. Arrows indicate areas of tumour invasion.

Figure 4.3. Systemic dissemination of U87MG-F-Luc cells in brain, blood and lung.

A-B, 5mg explant tissue or 5µL blood samples were homogenised and lysed for analysis of relative luciferase activity (R.L.U) and compared to control tissue. Luciferase background is 18 R.L.U for brain tissue and 12 R.L.U for blood and lung tissue. C-D, Cell number was quantified using titration curves for each tissue source (Figure 1). Data is the mean of

triplicates $\pm$ SEM, n=4 (A&C) and the mean of 3 independent tissue samples or 8 independent blood samples per mouse, n=4 (B&D). n=10 independent samples for control tissue and blood. *P<0.05, ****P<0.0001; unpaired student's t test.

Figure 4.4. Systemic dissemination of MU20-F-Luc cells in brain, blood and lung.

A-B, 5mg explant tissue or 5 μ L blood samples were homogenised and lysed for analysis of relative luciferase activity (R.L.U) and compared to control tissue. Luciferase background is 18 R.L.U for brain tissue and 8 R.L.U for blood and lung tissue. C-D, Cell number was quantified using titration curves for each tissue source (Supplementary Figure 1). Data is the mean of triplicates $\pm$ SEM, n=4 (A&C) and the mean of 3 independent tissue samples or 8 independent blood samples per mouse, n=4 (B&D). n=10 independent samples for control tissue and blood. *P<0.05, **P<0.01, ****P<0.0001; unpaired student's t test.

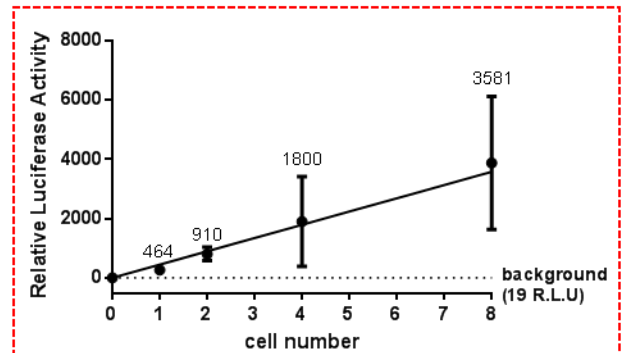
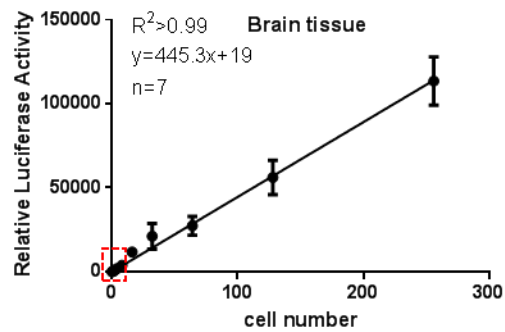
Figure 4.5. Systemic dissemination of U87MG-G-Luc cells in brain and lung 15 days after implantation.

A-B, 10mg explant tissue or 5 μ L blood samples were homogenised and lysed for analysis of relative luciferase activity (R.L.U) and compared to control tissue. Luciferase background was 115 R.L.U for brain tissue, 45 R.L.U for lung tissue and 8 R.L.U for blood. C-D, Cell number was quantified using titration curves for each tissue source (Supplementary Figure 1). Data is the mean of triplicates $\pm$ SEM, n=5 (A&C) and the mean of 3 independent tissue or blood samples per mouse, n=5 (B&D). n=10 independent samples for control tissue and blood. *P<0.05, ****P<0.0001; unpaired student's t test.

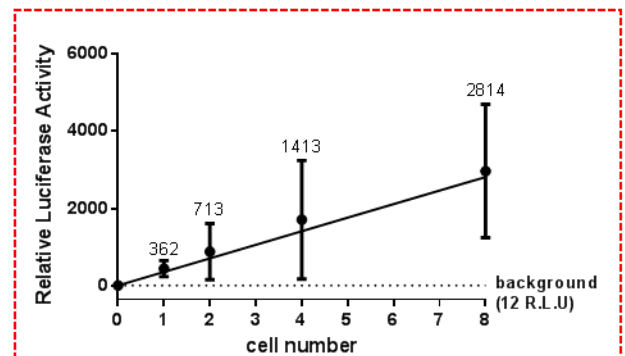
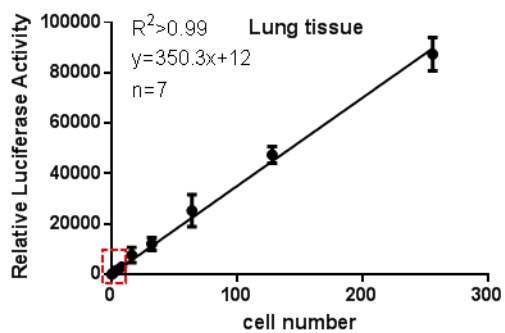
Figures

Figure 4.1.

A



B



C

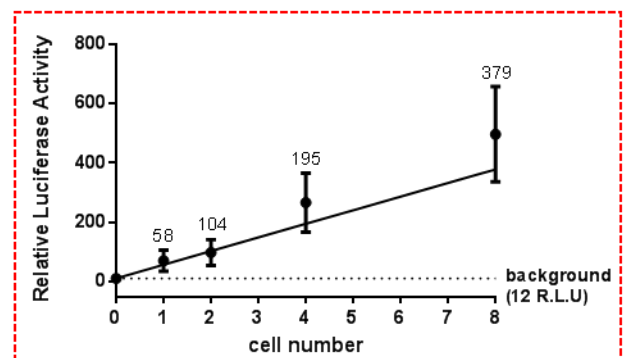
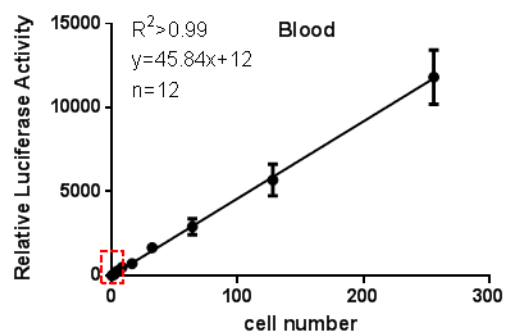


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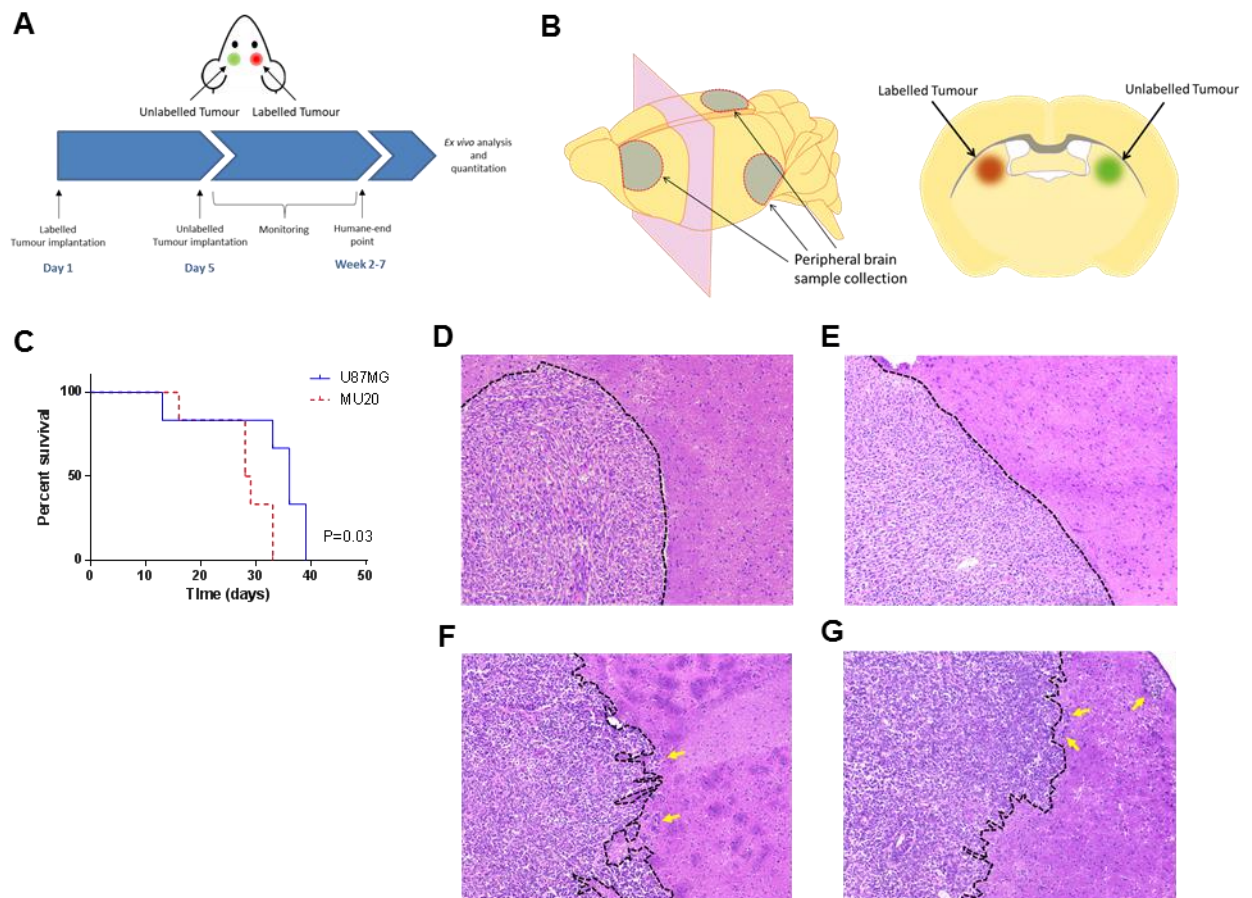
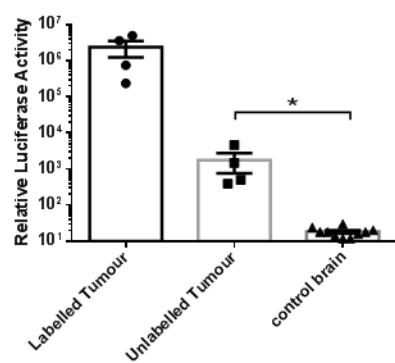
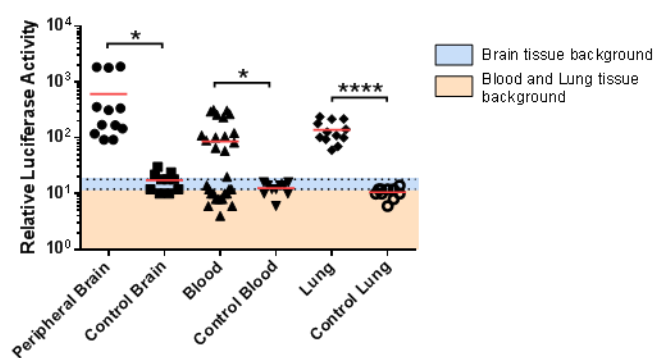


Figure 4.3.

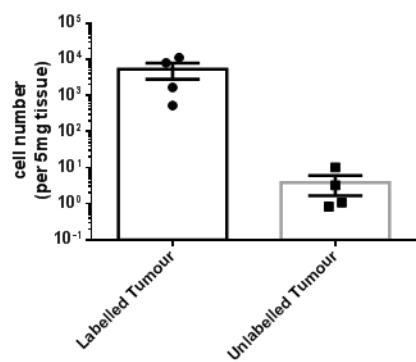
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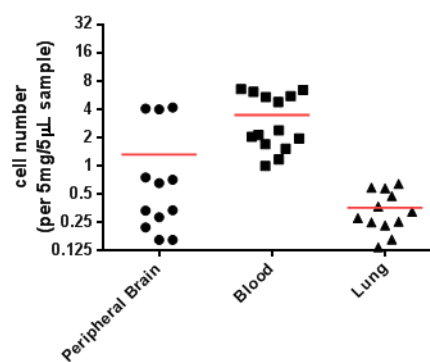


Figure 4.4.

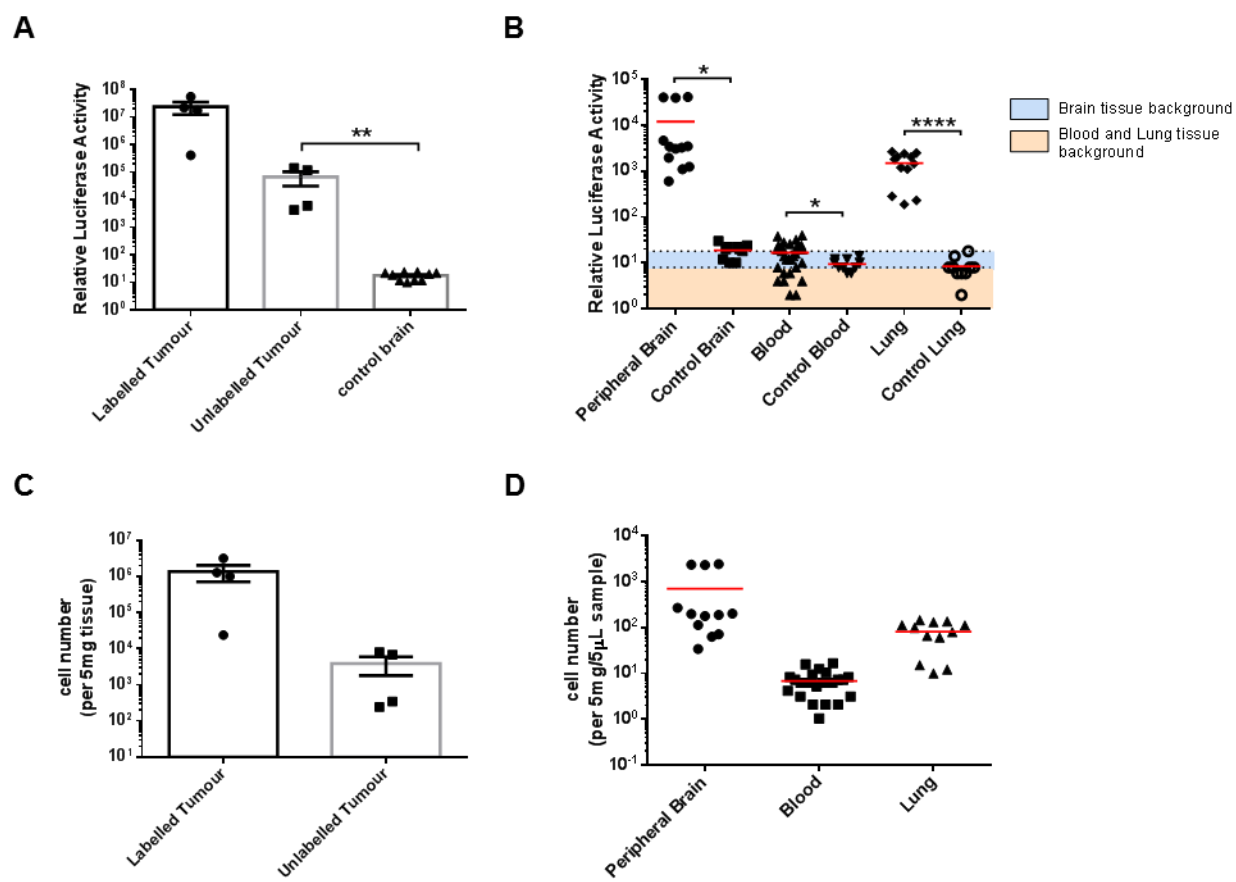
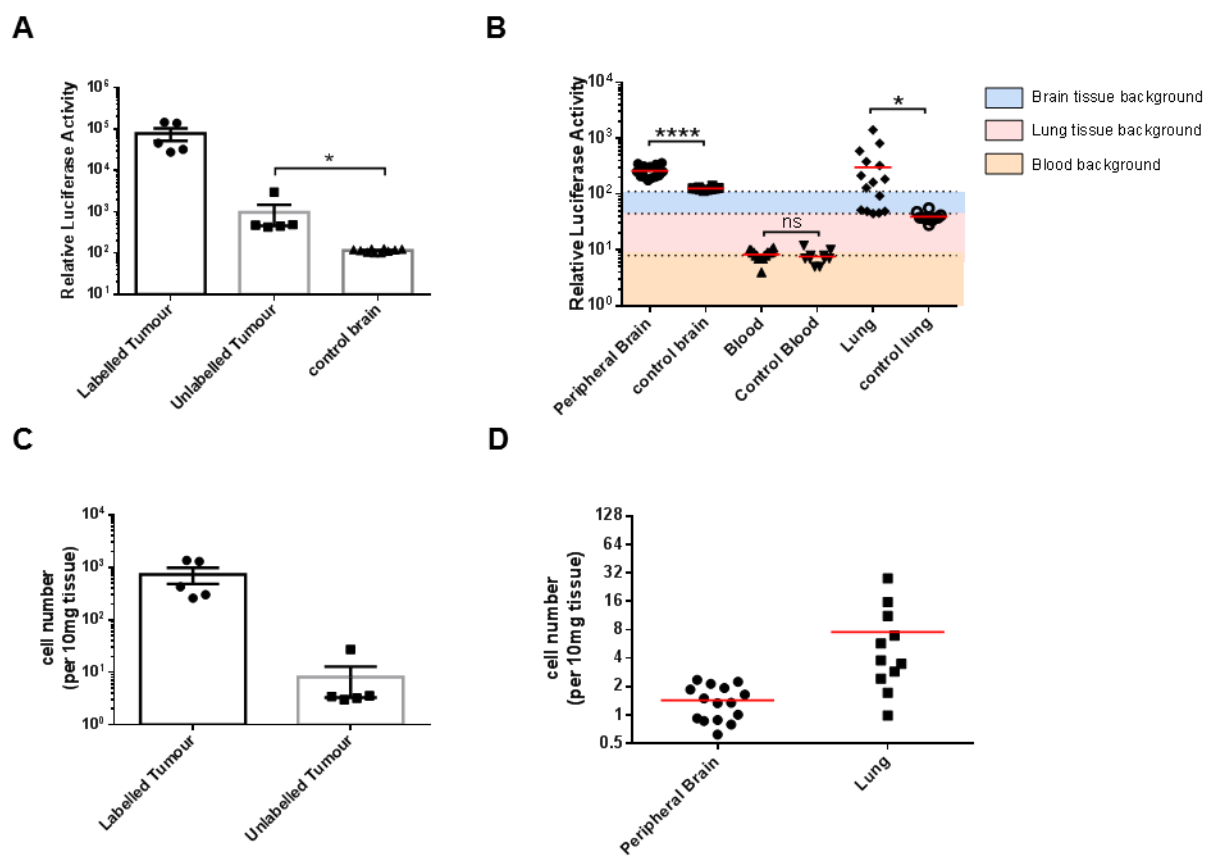


Figure 4.5.



Supplementary Figure Legends

Supplementary Figure 4.1. Standard curves for tumour cell quantitation for MU20-F-Luc and U87MG-G-Luc tumour cells.

MU20-F-Luc (A-C) and U87MG-G-Luc (D-F) tumour cells were serially diluted to single cells and spiked with brain tissue (A and D), lung tissue (B and E) or blood (C and F). Cells were lysed and relative luciferase activity (R.L.U) measured. Cell quantitation was determined by best-fit linear regression analysis. Serial titrations were performed in 5mg tissue or 5 μ L blood for MU20-F-Luc tumour cells (A-C), n=7, and 10mg tissue or 5 μ L blood for U87MG-G-Luc tumour cells (D-F), n=6. Data is mean $\pm$ SEM.

Supplementary Figure 4.2. Circulating tumour cell detection varies between mice and over time.

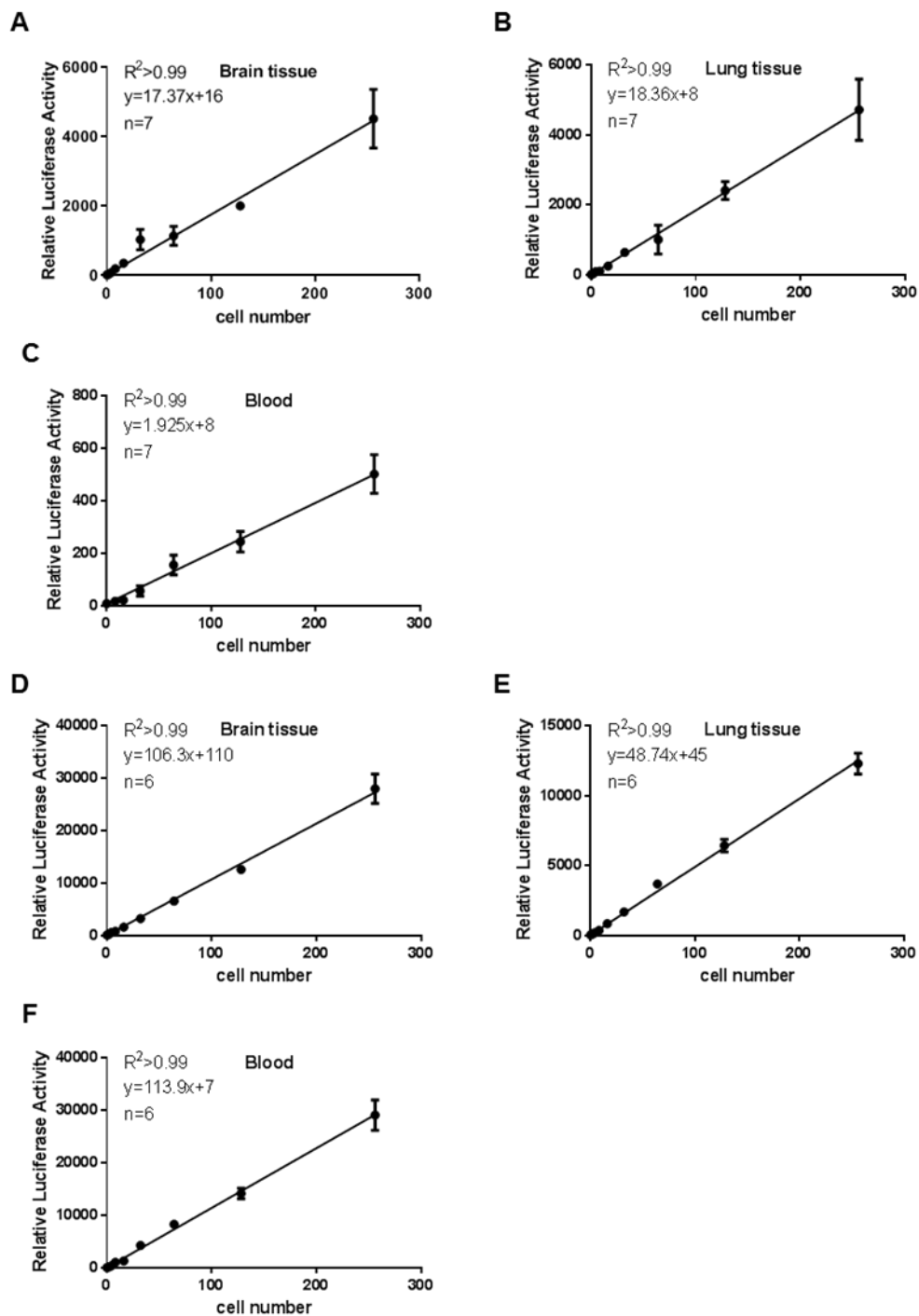
Mice were implanted with U87MG-F-Luc (A) or MU20-F-Luc (B) GBM tumours. Following euthanasia, blood was collected from the jugular vein and analysed for luciferase activity in 5 μ L aliquots. Data represents the mean of n=8 independent samples per mouse.

Supplementary Figure 4.3. GBM tumour cell detection in organs of mice orthotopically implanted with U87MG-F-Luc and MU20-F-Luc labelled cells.

Mice were implanted with U87MG-F-Luc (A) or MU20-F-Luc (B) GBM tumours. Following euthanasia, organs were homogenised and lysed for luciferase analysis. Data represents the mean of 3 independent 5mg tissue samples per mouse, n=4.

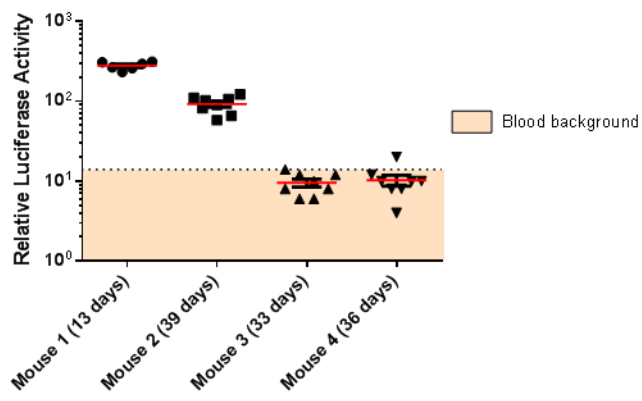
Supplementary Figures

Supplementary Figure 4.1.

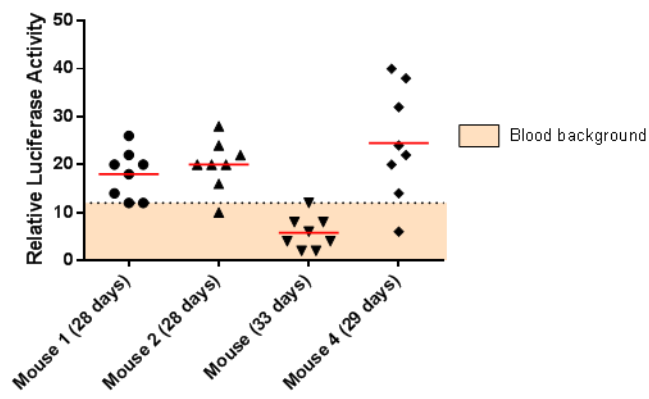


Supplementary Figure 4.2.

A

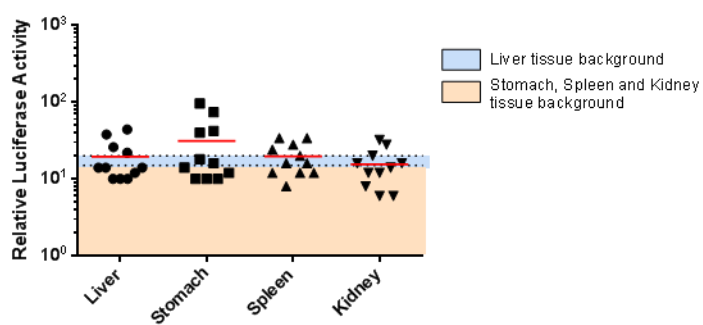


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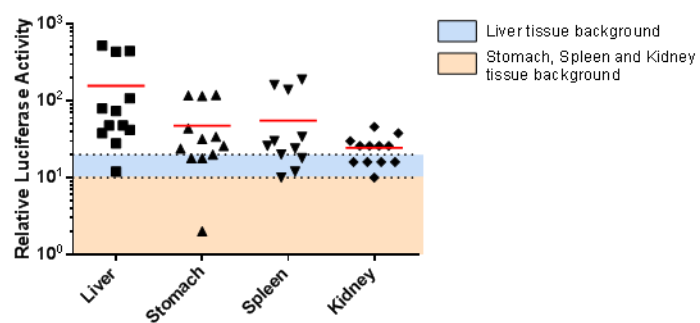


Supplementary Figure 4.3.

A



B



Chapter 5: TGF- β -ALK1-Smad1/5 signalling regulates glioblastoma endothelialisation and systemic brain dissemination

Key Points

Question Can tumour cells act like endothelial cells? What molecular mechanism regulating glioblastoma cell endothelialisation and what implications does it have for tumour progression?

Findings TGF- β -ALK5-SMAD2/3 signalling is expanded to activate ALK1-SMAD1/5 in glioblastoma cells and is a regulator of tumour cell endothelialisation. *In vivo*, inhibition of Smad1/5 signalling reduced tumour cell incorporation into the tumour vasculature and decreased whole brain tumour cell infiltration.

Conclusion Tumour cell endothelialisation promotes tumour progression through whole brain infiltration via haematogenous dissemination and is a novel biology for tumour recurrence.

5.1 Abstract

Importance Despite nearly two thousand clinical trials, no new advancements have been made to glioblastoma therapeutic care in the past 15 years. Of the most extensively tested agents, antiangiogenic inhibitors targeting the tumour vasculature have failed to produce any impact on patient survival, despite confirmed on-target effects. The discovery of vasculogenic mimicry in glioblastoma provides a novel perspective for the tumour vasculature, though the mechanisms regulating this phenomenon are still unknown.

Objective To determine the functional relevance and mechanisms regulating tumour cell endothelialisation.

Design, Setting and Participants The human glioblastoma cell lines U87MG, U118MG, MU20 and MU41 were used in this study. For *in vivo* experiments a novel orthotopic contralateral xenograft mouse model was used allowing for single tumour cell detection within the brain and lung tissue. Human glioblastoma patient *in situ hybridisation* sections were obtained from the Ivy Glioblastoma Atlas Project.

Results U87MG, U118MG and MU41, but not MU20 cells, formed lattice structures similar to endothelial cells when seeded onto matrigel *in vitro*. U87MG and MU41 cell lines were responsive to TGF- β ligand stimulation, activating the Smad1/5 signalling pathway, however, U118MG and MU20 cells did not show any activation with stimulation. When seeded onto Matrigel U87MG, U118MG and MU41 all activated the Smad1/5 signalling pathway. Inhibition of Smad1/5 signalling by overexpression of inhibitory Smad6 or ALK1 knockdown, disrupted lattice formation on Matrigel. *In vivo* U87MG implanted tumours showed considerable endothelialisation of tumour cells with incorporation into the tumour vasculature, which was inhibited by intracranial treatment with Ad-Smad6. Furthermore, U87MG tumours exhibited whole brain infiltration at 15 days post-implantation despite showing no invasive margins at the primary tumour. Intracranial treatment with Ad-Smad6 significantly reduced whole brain tumour dissemination.

Conclusions and Relevance The existence of vasculogenic mimicry has remained controversial due to a lack of identification of molecular mechanisms regulating this behaviour. This study has identified TGF- β -ALK1-Smad1/5 as a regulator of glioblastoma endothelialisation and vasculogenic mimicry formation. Functionally we have also determined that endothelialisation behaviour leads to whole brain tumour dissemination and

is a possible target for early recurrence prevention. These findings provide a new understanding of glioblastoma biology that can be exploited for progression-specific therapeutic targets.

5.2 Introduction

Glioblastoma is a WHO grade IV primary brain tumour and one of the most debilitating forms of human cancers.³⁸³ Despite an aggressive standard of care consisting of maximal surgical resection and radiochemotherapy, glioblastoma inevitably recurs resulting in a dismal median overall survival of 12-14 months.⁹¹ While the current standard of care has provided a small improvement to overall survival, prognosis remains disconcertingly similar to survival rates reported over 50 years ago.⁴⁴² The stagnation in glioblastoma survival has led to a plethora of new research that has significantly advanced our understanding of the molecular and genetic characterisation of glioblastoma and led to the development of new targeted therapies.^{21-23,388,443} Despite these extensive research efforts, no putative targets have translated successfully into clinical trials.³⁸⁸ More concerning the current success rate for any clinical trials showing improved survival remains nearly at 0%, an extremely disproportionate outcome compared to clinical trials in other aggressive low survival cancers. This is often despite evidence that therapies are on-target in treated patients, suggesting that current targets are not the key drivers of glioblastoma progression.^{388,428}

Current therapeutic strategies are failing to provide much needed improvement to glioblastoma survival, it is therefore important to re-examine our understanding of glioblastoma to identify new biology that can be exploited for therapeutic targeting. Due to the extensive and unique vascularity observed in glioblastoma, antiangiogenic inhibitors were once considered to be a significant breakthrough in the treatment of glioblastoma.⁴⁴⁴ Despite over a decade of research and clinical research showing that anti-VEGF inhibitor Bevacizumab was on-target resulting in normalisation of the tumour vasculature, clinical trials failed to demonstrate any overall survival benefit in both newly and recurrent glioblastoma.^{99,170,176} Furthermore, other antiangiogenic inhibitors tested in clinical trials also

did not confer a survival advantage suggesting that the original assumptions of the relevance of angiogenesis in glioblastoma may have been misappropriated.⁴⁴⁵

Close examination of the tumour vasculature reveals abnormalities in blood vessel structures, which are often dilated with large gaps in the endothelium walls.²³⁴ Along with these aberrant vasculature structures Mandiotis and colleagues discovered the presence of tumour cells incorporated within the endothelium of melanoma tissue specimens, which was later termed vasculogenic mimicry.²⁵¹ The presence of vasculogenic mimicry has been found in most solid cancers and has been associated with tumour metastasis and cancer progression.³⁴⁴ Despite these reports however, the existence of vasculogenic mimicry remains a controversial topic due to no known mechanisms regulating this behaviour.

Glioblastoma tumours report the highest incidence of vasculogenic mimicry structures (65.9%) and its presence is associated worse survival outcomes.^{344,348} Recently it was shown that treatment with the TGF- β signalling inhibitor galunisertib was able to attenuate vasculogenic mimicry structures in a glioblastoma xenograft model.²⁵⁶ TGF- β is a vital signalling pathway for the function of normal endothelial cells consisting of TGF- β -Smad2/3 and endothelial cell specific TGF- β -Smad1/5 signalling.^{446,447} Glioblastoma tumour cells have recently also been reported to contain the expanded TGF- β -Smad1/5 required for endothelial cell function, though functional relevance of this expansion remains unknown in glioblastoma.³⁸¹

This study aims to identify the molecular pathway relating the transition of tumour cell to endothelial-like phenotypes and vasculogenic mimicry formation. We will also explore the functional biology of this phenomenon and its relevance for glioblastoma tumour progression.

5.3 Materials and Methods

Cells and cell culture

Human glioblastoma cell lines MU20 and MU41 were derived from patients at the Royal Melbourne Hospital (Parkville) and U87MG obtained from the ATCC (American Type Culture Collection). All cells were maintained in Dulbecco's modified Eagle's medium and contained 10% fetal bovine serum (FBS) (Thermo Fischer Scientific, USA), 2mM glutamine, 100U/ml penicillin and 100µl/ml streptomycin (Invitrogen, USA). Cells were incubated in a humidified atmosphere of 10% CO₂ 37°C.

Antibodies and reagents

Goat polyclonal antibodies anti-smad6, anti-smad7 and anti-smad5 were purchased from Santa Cruz Biotechnology (sc6034, 1:1000; sc7004, 1:1000; sc7443, 1:1000). Mouse monoclonal anti-smad2/3 antibody was purchased from Transduction Laboratories (S66220, 1:1000). Rabbit polyclonal anti-phospho-smad2/3 and anti-phospho-smad1/5 antibody was gifted from Prof. Peter Ten Dijke (Leiden University Medical Center, Netherlands). Monoclonal mouse anti-actin antibody was purchased from Sigma-Aldrich (A-4700, 1:1000). HRP-conjugated secondary antibodies (1:5000) were purchased from Bio-Rad.

Human recombinant TGFβ1 and human recombinant BMP7 was purchased from PeproTech (100-21-100; 120-03-10). Adenovirus vectors Ad-CMV-Tomato, Ad-BRE-Tomato, Ad-BRE-Firefly-Luciferase, Ad-CAGA-Gussia-Luciferase, Ad-CMV-Smad6 and Ad-CMV-Smad7 were all generated by the Zhu lab. FuGENE® HD transfection reagent was purchased from Promega (E2312).

Western blotting analysis

Cells were lysed in lysis buffer (30mM HEPES, 1% Triton-X-100, 2mM MgCl, 150mM NaCl, 5mM EDTA, complete protease inhibitor cocktail (Roche, Switzerland) on ice for

30mins. Lysates were centrifuged at 13000rpm for 15mins and supernatant extracted. Lysates were heated at 95°C for 10 minutes Laemmli sample buffer (Bio-Rad Laboratories, Australia). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Australia). After blocking with 4% skim milk for 1h at room temperature, membranes were probed with primary and secondary antibodies. The signal was developed using Western Lightning® ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer, USA). All western blotting results were representative of three separate experiments.

Adenovirus fluorescence and immunofluorescence

Cells were transduced with Ad-CMV-Td-Tomato containing virus in 5% FBS DMEM media. Optimal expression was provided after 48hr infection.

For *in vitro* immunofluorescence cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, USA) for 5-7mins and blocked in 1% BSA for 1hr at room temperature. Cells were probed with primary and ALEXA fluorescence secondary antibodies. Fluorescence signal was detected using a Leica fluorescent microscope.

For tissue section immunofluorescence, fresh frozen tissue is sectioned at 6microns and fixed in 100% acetone at -20°C for 10mins. Sections are blocked in 5% normal serum and probed with primary and ALEXA secondary antibodies. Slides are stained with 1 µg/µL Hoescht for 5mins. Sections are mounted using VectaShield Antifade Mounting Media for Fluorescence (Vector Laboratories, USA) and sealed using clear nail polish. Fluorescent signal was detected using Leica fluorescent microscope and Leica sp5 Confocal microscope.

Luciferase assay

Reporter cells (5000 cells/well in 96-well plate) were seeded and transduced with Ad-BRE-Firefly-Luciferase virus (MOI: 250) and Ad-pCAGA-Gaussia-Luciferase virus (MOI: 100) in

5% FBS DMEM media. After 24hr infection, cells were serum starved in 100µl serum free DMEM media for 16hrs. Reporter cells were stimulated with indicated ligand (TGF-β or BMP7) for a further 6hrs. Thereafter, cells were lysed and assessed for luciferase activity using Luciferase Assay Kit (Promega, USA) following the manufacturer's instructions. Luciferase activity was presented as the fold change as compared with control.

***In vitro* lattice formation assay**

48-well tissue culture plate were coated with Matrigel Basement Membrane Matrix (100µl/well, BD Bioscience, Australia), which was polymerised at 37°C for 1hr. Cells were resuspended and seeded on Matrigel at 4.5×10^4 cells/ml in 5% FCS DMEM media and incubated for 6hrs at 37°C. To investigate the effects of inhibitory Smads on lattice formation, cells were transduced with Ad-CMV-Td-Tomato, Ad-Smad6 and Ad-Smad7 48 hrs prior to seeding. Cultures were photographed using a Leica fluorescence microscope. Images were quantified using angiogenesis analyzer

<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>.

Stable transfection of firefly and gaussia luciferase

U87MG cells were stably transfected with pGL4.51[luc2/CMV/Neo] (Promega, NSW, Australia) or pCMV-Gluc-KDEL (Prolume Ltd., USA) vector using Fugene® HD (Promega, Australia). Cells underwent selection with 2mg/ml G418 (Roche, Switzerland) and stable clonal colonies obtained.

Animal experimentation

Animal experimentation has been approved by the University of Melbourne Animal Ethics Committee (1614055). 6-8 week female NOD-SCID mice were purchased from Animal Resource Centre (ARC) Canning Vale, Western Australia and housed in the Department of Surgery Animal Facility, University of Melbourne, Victoria. For intracranial implantation,

mice were anaesthetized with 4% isoflurane and maintained at 2% isoflurane for the procedure. Mice were shaved and positioned on the stereotactic. A small burr hole <1mm diameter was fashioned and 5×10^4 firefly-labelled cells implanted in 5µL using a 27-gauge needle at a flow rate of 1µL/min. The needle was retracted and the hole sealed using sterile bone wax. Mice were monitored for recovery and 5 days later a non-labelled contralateral tumour was implanted using the same procedure. Once recovered mice were transferred to Monash Institute of Pharmaceutical Sciences for IVIS imaging and blood collection. 150mg/kg D-luciferin was i.p injected into mice for IVIS illumina II (Caliper Life Sciences, USA) bioluminescent detection.

Following experimental end-point, mice are euthanized and brains harvested. The firefly-labelled tumour is frozen in OCT and sectioned for immunofluorescence. The unlabeled tumour is lysed in cell culture lysis buffer (Promega, USA) and luciferase bioluminescence measured on a luminometer.

Human glioblastoma images

Human glioblastoma images of *in situ* hybridization and H&E tissue sections were obtained from the publicly available Ivy Glioblastoma Atlas Project (Ivy GAP) (<https://glioblastoma.alleninstitute.org/>).

Statistical analysis

All experiments were determined three times. The data were recorded as mean $\pm$ SEM and statistical analysis was performed using Graphpad Prism 6. Differences between groups were analysed by one-way ANOVA with Tukey's multi-comparisons test or using the student's t test. P value < 0.05 was considered statistically significant.

5.4 Results

Investigating the lattice formation properties of glioblastoma cell lines

Vasculogenic mimicry describes the ability of tumour cells to mimic endothelial cell behaviour.²⁵¹ In order to establish the mechanisms regulating tumour endothelialisation, we investigated the lattice formation properties of glioblastoma cell lines. When seeding cells on wells coated with the basement membrane surrogate matrix Matrigel *in vitro*, we observed lattice formation for U87MG, U118MG and MU41 cell lines, recapitulating endothelial cell behaviour (Figure 5.1A). The MU20 cell line, however, did not form any lattice structures, presenting with a spherical and undifferentiated-like morphology. To measure the intricacy of these lattice structures we utilised the ImageJ macro – *angiogenesis analyzer*, allowing for quantitation of key lattice characteristics (Figure 5.1B). Using this quantitative method, we described the complexity of lattice formations for each cell line (Figure 5.1C). U87MG, U118MG and MU41 cell lines all displayed increased inter-cell-connectivity when seeded onto Matrigel as measured by an increase in the number of junctions per field ([Control vs Matrigel]: U87MG, 5.3 ± 1.2 vs 32.3 ± 0.9 ; U118MG, 4.0 ± 2.6 vs 36.7 ± 3.0 ; MU41, 5.7 ± 0.9 vs 36.3 ± 4.3 ; $P < 0.0001$) leading to the formation of mesh-like structures ([Control vs Matrigel]: U87MG, 0.0 ± 0.0 vs 14 ± 0.5 ; U118MG, 0.0 ± 0.0 vs 15.3 ± 1.7 ; MU41, 0.7 ± 0.7 vs 15 ± 1.7 ; $P < 0.0001$). Elongation of individual processes was also observed in the Matrigel coated wells compared to control contributing to an increase in total cell length ([Control vs Matrigel]: U87MG, 1910 ± 95.3 pixels vs 3295 ± 83.8 pixels, $P < 0.001$; U118MG, 1648 ± 361.2 pixels vs 3184 ± 151.6 pixels; MU41, 1637 ± 22.0 pixels vs 3265 ± 37.9 pixels; $P < 0.0001$). In contrast, MU20 cells did not show any inter-cell-connectivity in either the control or Matrigel coated wells and did not exhibit any morphological change.

TGF- β -Smad1/5 signalling is expanded in glioblastoma cell lines

Having established endothelial cell-like mimicry in some of our glioblastoma cell lines, we next sought to investigate the underlying mechanisms regulating this behaviour. A recent study reported decreased vasculogenic mimicry formation when glioblastoma xenografts were treated with the ALK5 kinase inhibitor Galunisterib, targeting the TGF- β signalling pathway.²⁵⁶ TGF- β signalling has been shown to be vital for the normal function of endothelial cells and blood vessel formation and stabilisation.³⁶² This is performed via two canonical signalling pathways: the common TGF- β -ALK5-Smad2/3 pathway and the endothelial specific TGF- β -ALK1-Smad1/5 pathway. Recently it was reported that some glioblastoma tumour cells have acquired TGF- β -Smad1/5 signalling, though the receptor mediating this activation and its relevance remain unclear.³⁸¹ To determine if TGF- β -Smad1/5 may be involved in tumour endothelialisation we investigated whether the pathway was activated in our glioblastoma cell lines. Using a Smad1/5 specific promoter BRE-luciferase reporter, we confirmed TGF- β dose-dependent activation of the BRE-mediated transcription in U87MG cells, which plateaued at ~2ng/mL with an ~4-fold increase over baseline untreated levels (Figure 5.2A). Similarly, the Smad2/3 specific promoter CAGA₁₂ was also activated in a TGF- β dose-dependent manner reaching a signal plateau at 2ng/mL. Western blot analysis also showed increased phosphorylation in a time-dependent manner with 2ng/mL TGF- β stimulation in U87MG cells confirming activation of the TGF-Smad1/5 signalling pathway (Figure 5.2B). Having confirmed expansion of the TGF- β -Smad1/5 signalling pathway in U87MG cells we tested the rest of our glioblastoma cell lines for Smad1/5 signalling activation. Both U87MG and MU41 cells showed over 2-fold increases in BRE-luciferase reporter activity in response to 2ng/mL TGF- β activity and 20ng/mL BMP7 (positive control) (Figure 5.2C). Interestingly, BRE-luciferase activity remained unchanged in U118MG cells with 2ng/mL TGF- β stimulation, although U118MG cells were responsive to 20ng/mL BMP7. In contrast, U87MG, U118MG, and MU41 all displayed increased

CAGA-luciferase reporter activity with 2ng/mL TGF- β treatment. MU20 did not respond to either TGF- β or BMP7 stimulation. Western analysis further confirmed the results of the downstream luciferase reporters showing phosphorylation of Smad1/5 and Smad2/3 with TGF- β treatment in U87MG and MU41 cells, phosphorylation of only Smad2/3 in U118MG cells (Figure 5.2D). While MU20 cells again did not respond to TGF- β or BMP7 treatment though both Smad1/5 and Smad2/3 proteins appear to be present within the cells. When seeded on Matrigel U87MG, U118MG and MU41 showed significant increases of 2-fold and above for BRE-luciferase reporter activity compared to uncoated wells, while MU20 reporter activity remained unresponsive (Figure 5.2E).

TGF- β -ALK1-Smad1/5 signalling regulates lattice formation of glioblastoma cell lines *in vitro*

Having confirmed the expansion of the TGF- β -Smad1/5 signalling pathway in the lattice forming glioblastoma cell lines, we sought to specifically inhibit the pathway to investigate its functional relevance on tumour endothelialisation. We utilised adenoviruses to efficiently transduce tumour cells with the specific Smad1/5 inhibitor Smad6 (Ad-Smad6-M2) or the Smad1/5 and Smad2/3 inhibitor Smad7 (Ad-Smad7-M2). Smad6 and Smad7 expression increased with adenovirus multiplicity of infection (MOI) with clear inhibition of Smad1/5 phosphorylation even at the lowest MOI of 500 in U87MG cells (Figure 5.3A).

Immunofluorescence probing for M2 confirmed 100% infection rate for both Ad-Smad6-M2 and Ad-Smad7-M2 at 1000 MOI in U87MG cells (Figure 5.3B). 100% adenovirus transduction efficiency was also achieved in U118MG, MU20 and MU41 cells at 1000 MOI (Supplementary Figure 3.1). Using the BRE-and CAGA-luciferase reporter, we next confirmed the specificity and sensitivity of Ad-Smad6 and Ad-Smad7 viruses for the TGF- β signalling pathways. 1000 MOI transduction of Ad-Smad6 specifically inhibited TGF- β and BMP7 mediated activation of the BRE-luciferase reporter but did not affect CAGA-luciferase

reporter activity in U87MG, U118MG and MU41 cells (Figure 5.3C-D&F). 1000 MOI transduction with Ad-Smad7 also inhibited activation of the BRE-luciferase reporter for both TGF- β and BMP7 stimulation and also inhibited TGF- β mediated activation of the CAGA-luciferase reporter. Together these results confirm Ad-Smad6 as a specific inhibitor of Smad1/5 signalling, while Ad-Smad7 is both inhibitory to Smad1/5 and Smad2/3 activation. MU20 cells did not show any change to BRE- and CAGA-luciferase reporter with transduction of either 1000 MOI Ad-Smad6 or Ad-Smad7.

Transduction with 1000 MOI Ad-Smad6 and Ad-Smad7 caused significant disruption to lattice formations in U87MG, U118MG and MU41 cells compared to control Ad-CMV-Tom (Figure 5.4A). Total cell length significantly decreased in Ad-Smad6 treated U87MG and MU41 cells ([Ad-CMV-Tom v Ad-Smad6]: U87MG, 3006 ± 129.7 pixels v 2285 ± 79.1 pixels; MU41, 3785 ± 119.5 pixels v 2725 ± 127.9 pixels; $P < 0.01$) but not U118MG (Figure 5.4B). Ad-Smad7 treatment, however, decreased total cell length in U87MG ($P < 0.01$), U118MG ($P < 0.001$) and MU41 cells ($P < 0.001$). MU20 cells, already presenting with spherical morphology did demonstrate any change in cell length. Both Ad-Smad6 and Ad-Smad7 treatment attenuated the number of meshes and junction formed for U87MG, U118MG and MU41 cells. MU20 did not form any meshes or junctions even within the control Ad-CMV-Tom treated cells and graphs for these characteristics were not presented. Following image capture for lattice disruption, on-target inhibition of Smad1/5 was confirmed for each treatment group (Figure 5.4C). Ad-Smad6 and Ad-Smad7 demonstrated specific inhibition of BRE-luciferase activity compared to control uncoated wells in U87MG, U118MG and MU41 at time point lattice formation images were captured. MU20 cells did not demonstrate any increased BRE-luciferase activity when seeded on Matrigel nor responded to Ad-Smad6 or Ad-Smad7 treatment.

Having confirmed a functional relevance for Smad1/5 signalling in glioblastoma endothelialisation behaviour we next further investigated the activation of this pathway. Since Smad1/5 can be activated by both TGF- β and BMP ligands we first sought to establish the specificity of TGF- β signalling in glioblastoma lattice formation. We utilised the small molecular inhibitor SB-431542, an inhibitor of TGF- β but not BMP type 1 receptor. Treatment with 10 μ M SB-431542 prevented TGF- β mediated activation of both BRE- and CAGA-luciferase reporter compared to control DMSO treatment (Figure 5.5A). Additionally, SB-431542 treatment completely abolished lattice formation in U87MG, preventing the formation of any junctions or meshes and significantly reducing total length of cells (Figure 5.5B&C).

In endothelial cells ALK1 receptor is the mediator of TGF- β -SMAD1/5 activation, although this pathway has not been described in glioblastoma tumour cells. To test the function of ALK1 in SMAD1/5 signalling we generated stable shRNA-ALK1 clonal knockdowns using 3 different shRNA sequences (shRNA: 354, 355, 356) in U87MG cells. Western blot analysis confirmed successful knockdown of shRNA-ALK1 354, 355 and 356 in comparison to vector control, with shRNA-ALK1 356 displaying the most efficient knockdown. Inhibition of TGF- β mediated BRE-luciferase activity was observed only in shRNA 356 compared to vector control (Figure 5.6B). When seeded onto Matrigel lattice formation was observed in vector, 354 and 355 clones but not 356 (Figure 5.6C). Clone 356 displayed over 50% decrease in junction number and total length of cells with almost complete abrogation of mesh-like structures (Figure 5.6D). Clone 354 and 355 did not show any difference compared to vector control. Specific inhibition of BRE-luciferase activation was also confirmed in clone 356 when seeded onto Matrigel compared to vector control and clone 354/355 (Figure 5.6E).

To further support the role of ALK1-Smad1/5 signalling in glioblastoma endothelialisation we generated an adenovirus delivering constitutively active ALK1 receptor (Ad-caALK1).

Successful overexpression of ALK1 receptor in MU20 cells by Ad-caALK1 transduction was confirmed via western blot. Transduction with Ad-caALK1 resulted in significant activation of the BRE-luciferase reporter and to a lesser degree CAGA-luciferase reporter in the TGF- β non-responsive glioblastoma cell line MU20 (Figure 5.7A). Expression of caALK1 in MU20 cells induced patterned lattice formation structures compared to CMV-Tom control, which formed large indiscriminate clumping of cells (Figure 5.7B). The number of cell junctions and lattice meshes significantly increased in the Ad-caALK1 transduced cells compared to control (Figure 5.7C). Remarkably Ad-caALK1 transduced MU20 cells also showed some elongation in cell morphology leading to increased total length of lattices compared to control.

***In vivo* inhibition of Smad1/5 signalling reduces glioblastoma endothelialisation and whole brain tumour dissemination**

Our previous *in vitro* results established the capacity of U87MG cells to undergo endothelialisation, while MU20 lacked this ability. We therefore sought to investigate the endothelialisation potential of these two contrasting cell lines *in vivo*. Using a mouse xenograft model we observed different growth rates between U87MG and MU20 tumour implants. MU20 tumours grew faster and were comparatively larger in size than U87MG tumours by experimental end-point (Figure 5.8A). Despite the difference in growth rates, mouse body weights remained similar up until experimental end-points at around 26-32 days (Figure 5.8B). Interestingly, in addition to growth rate differences, U87MG tumours displayed expansive growth with no clear invasive margins while MU20 tumours exhibited extensive invasion at the primary tumour margin (Figure 5.8C). Immunofluorescence of U87MG tumours located human HLA-A staining around and within close proximity to CD31⁺ blood vessels (Figure 5.8D). The CD31⁺ blood vessels were found extensively throughout the primary tumour, characterised by dilated and irregular sized vessels.

Significantly these blood vessels displayed many gaps in CD31⁺ staining, suggesting abnormalities in the lining of the blood vessels. Close examination of these blood vessels identifies HLA-A⁺ cells lining these gaps (Figure 5.8D [zoomed in image]). We further quantified the pixel intensity of CD31 and HLA-A stains across the blood vessel circumference and identified a clear absence of CD31 staining from numbers 2-3[zoomed image], which was replaced by HLA-A⁺ staining suggesting a change in the markers expressed within this part of the blood vessel. CD31⁺ staining was also observed to be highly variably in expression intensity across the blood vessel further establishing the irregularity of these blood vessels. Immunofluorescence of MU20 tumour in contrast displayed relatively few blood vessels which were markedly smaller in size (Figure 5.8E). HLA-A staining was still extensively observed across the tumour section, however, CD31⁺ blood vessels were less variable in their observed intensity and HLA-A⁺ staining was not found within the blood vessel circumference (Figure 5.8E [zoomed in image]). In comparison to MU20 tumours, U87MG tumours presented with increased blood vessels per field (U87MG: 22 ± 2 , MU20: 8 ± 1 ; $P < 0.01$) (Figure 5.8F). We did not observe any endothelialisation within MU20 tumour, however, endothelialised cells were observed in 14% of U87MG tumour blood vessels, with an average of 33% of cells from tumour origin with these endothelialised blood vessels. Overall, 16% of observed blood vessel cells were observed to be endothelialised tumour cells incorporated into the tumour vasculature.

To determine if Smad1/5 signalling was regulating endothelialisation and the functional consequences of this phenomenon we utilised a contralateral orthotopic xenograft mouse model by implanting BRE-firefly-luciferase and CMV-gaussia-luciferase dual-labelled U87MG cells into one hemisphere, and unlabelled wild-type U87MG into the other hemisphere (Figure 5.9A). At day 9 post-tumour implantation mice were intracranially treated with either Ad-CMV-Tom, Ad-Smad6 or Ad-Smad7. The body weights of all mice

were monitored over the course of the experiment and did not significantly change between treatment groups (Figure 5.9B).

To confirm that Ad-Smad6 and Ad-Smad7 treatment was on-target we measured BRE-luciferase reporter activity prior and 2-days post intracranial treatment (Figure 5.9C). BRE-luciferase reporter activity was similar prior to treatment with Ad-Smad6 and Ad-Smad7 compared to control, however, there was a significant decrease in BRE-luciferase reporter activity 2-days following treatment (control: $1.14 \times 10^5 \pm 0.08 \times 10^5$ R.L.U ; Ad-Smad6: $0.73 \times 10^5 \pm 0.14 \times 10^5$ R.L.U; Ad-Smad7: $0.65 \times 10^5 \pm 0.09 \times 10^5$ R.L.U; $P < 0.05$).

Ad-CMV-Td-Tomato and Ad-Smad6 treated mice displayed similar growth patterns with no evidence of invasive margins via histological analysis (Figure 5.9D). Tumours were not observed in the Ad-Smad7 treated group via histological analysis and sections are not shown. Immunofluorescence of tumour treated Ad-CMV-Tom demonstrated expansive and dense HLA-A⁺ tumour cells within close proximity to CD31⁺ blood vessels (Figure 5.9E). Blood vessels displayed variable CD31 expression intensity across blood vessels and presented with gaps in expression. Close inspection of these gaps confirmed the presence of HLA-A⁺ tumour cells lining the sections of the tumour vasculature (Figure 5.9E [zoomed in image]). Tumours treated with Ad-Smad6 also exhibited dense and expansive HLA-A⁺ tumour cells, which closely surrounded CD31⁺ blood vessels (Figure 5.9F). In contrast to Ad-CMV-Tom treated tumours, Ad-Smad6 treated tumours presented with less variable CD31⁺ expression intensity across blood vessels and presented with fewer/smaller CD31 marker gaps (Figure 5.9F [zoomed in image]). Although both Ad-CMV-Tom and Ad-Smad6 treated tumour exhibited a similar number of blood vessels per field, the percentage of blood vessels with endothelialised cells decreased from $11.5 \pm 2.0\%$ in control to $3.9 \pm 1.4\%$ in the Smad6 treated group ($P < 0.05$) (Figure 5.9G). The average number endothelialised cells within endothelialised blood vessels also reduced from $27.4 \pm 4.9\%$ to $11.0 \pm 3.8\%$ (control to

treatment; $P < 0.05$) and the percentage of endothelialised cells as a total of all blood vessel cells observed per field reduced from $14.0 \pm 3.3\%$ to $3.9 \pm 1.3\%$ (control to treatment; $P < 0.05$).

Having established a molecular mechanism successfully regulating endothelialisation in glioblastoma we next sought to discover the functional relevance of endothelialisation for tumour progression. Since tumour recurrence remains the most significant barrier to glioblastoma treatment, we utilised techniques developed by our lab to analyse the overall dissemination and infiltration of the mouse brain as a model for tumour recurrence. *Ex vivo* analysis of the primary labelled tumour revealed similar luciferase activity between Ad-CMV-Td-Tomato and Ad-Smad6 treated groups, which were significantly higher than background control brain luciferase readings (Figure 5.10A; left panel). Comparatively however, there was no statistical difference between Ad-Smad7 and background control brain consistent with the lack of observed tumour growth within this treated group. Since tumour cells were also labelled with BRE-firefly-luciferase reporter we also confirmed that the Ad-Smad6 treated tumour cells still maintained reduced BRE transcriptional activity. Indeed, Ad-Smad6 treated tumours still displayed diminished BRE-luciferase reporter activity in comparison to Ad-CMV-Tom tumours (Ad-CMV-Tom: 602 ± 101.6 R.L.U; Ad-Smad6: 212.6 ± 94.7 R.L.U, $P < 0.01$) (Figure 5.10A; right panel). Interestingly, analysis of the unlabelled contralateral tumour also displayed positive gaussian luciferase signal for all treatment groups compared to background control brain, (Ad-CMV-Tom: 448.8 ± 16.6 R.L.U; Ad-Smad6: 388.3 ± 23.4 R.L.U; Ad-Smad7: 326.8 ± 15.2 R.L.U; control brain: 109.1 ± 5.5 R.L.U; $P < 0.05$) (Figure 5.10B). Calculation of the cell numbers in the unlabelled tumours samples found 3.2 ± 0.2 cells/10mg in Ad-CMV-Tom treated mice, 2.6 ± 0.2 cells/10mg in Ad-Smad6 treated mice; 2.0 ± 0.1 cells/10mg in Ad-Smad7 treated mice ($P < 0.01$). Finally, we also detected tumour specific luciferase activity in peripheral brain

samples of all tumour implanted mice to background control brain ($P < 0.0001$; Figure 5.10C). The number of cells present in the peripheral brain were reduced, however, in Ad-Smad6 and Ad-Smad7 treated mice compared with Ad-CMV-Tom (Ad-CMV-Tom: 1.4 ± 0.1 cells/10mg; Ad-Smad6: 0.8 ± 0.1 cells/10mg; Ad-Smad7: 0.5 ± 0.0 cells/10mg; $P < 0.001$).

Endothelialised glioblastoma cells are present in human tumour sections

To confirm the presence of tumour endothelialisation occurs in human glioblastoma cases we obtained primary glioblastoma sections from the publicly available Ivy Glioblastoma Atlas Project (Ivy GAP), which provides a resource of *in situ* hybridisation (ISH) and adjacent hematoxylin and eosin (H&E)-stained tissue sections. *FGFR3-TACC3* is a tumour specific fusion-gene, which occurs in 1-6% of glioblastoma patients and is present within 2 patients within the Ivy GAP resource.^{448,449} Using *FGFR3-TACC3* as a tumour specific marker, we analysed blood vessels of the patients for the presence of tumour cells located within the tumour endothelium. Blood vessels were determined morphologically using H&E stained adjacent tissue sections (Figure 5.11 and Supplementary Figure 5.3; right panels). *FGFR3-TACC3* positive cells were found to be extensively incorporated into the blood vessels of both patients (Figure 5.11A and Supplementary Figure 5.3A). These *FGFR3-TACC3* positive cells in some cases appeared to elongate and stretch across the blood vessel, taking the form of an endothelial-like morphology (Figure 5.11A; middle panel). We next sought to characterise the integrity of the tumour vasculature using genes known to be enriched in tumour vasculature endothelial cells. *ESM1* expression was observed in almost all blood vessels, however, presented with significant gaps in circumferential staining (Figure 5.11B and Supplementary Figure 5.3C). Similarly, the endothelial enriched markers of *MECOM* and *ENPEP* also were also found to be highly expressed with the tumour vasculature, however, unstained cells were still present within blood vessels suggesting irregularities in cell presentation (Figure 5.11C&D and Supplementary Figure 5.3D&E).

To confirm that these irregularities are not specific to *FGFR3-TACC3* positive glioblastoma tumours, we analysed other patient sections within the Ivy GAP dataset. Gaps in the staining of endothelial enriched markers *ESM1*, *MECOM* and *ENPEP* were also found within the endothelium of patients negative for the *FGFR3-TACC3* fusion gene (Figure 5.12A-C; left and middle panel)). Some patients, however, displayed complete staining of these endothelial markers, with no gaps or unstained nuclei present (Figure 5.12A-C; right panel). To further confirm these findings, we next examined expression of *EPAS1*, which was not available for the *FGFR3-TACC3* patients and is highly enriched in endothelial cells of the tumour vasculature. *EPAS1* was found to be upregulated in most patient samples examined, yet unstained nuclei were still detected in most patient sections (Figure 5.12D; left and middle panel). Caution should be used in the interpretation of *EPAS1* as an endothelial marker since it can also be expressed by glioblastoma cells. Despite, this the lack of staining supports irregularities in the blood vessel structure. Lastly, we looked at *GFAP* as marker of tumour cells finding that *GFAP* positive cells were incorporated into all patients with available tissue sections for this marker (Figure 5.12E and Supplementary Figure 5.3B). While *GFAP* is expressed also by astrocytes in addition to glioblastoma cells, endothelial cells do not express this marker, which further describes the incorporation of non-endothelial cells into the tumour vasculature. Overall endothelial enriched markers detected gaps in the tumour vasculature ranging from 60%-87.5% of all cases, while *GFAP* staining was only available in 7 patients and was detected in the tumour vasculature of all patients (Table 5.1). Together these results provide evidence of specific tumour cell endothelialisation in the blood vessels of human glioblastoma patients and further characterise the inherent biological abnormality of the tumour vasculature.

5.5 Discussion

The existence of vasculogenic mimicry has remained a controversial issue ever since its first report by Maniotis and colleagues in 1999.²⁵¹ The field has since expanded to include alternate patterns of tumour cell – blood vessel interactions, however our understanding into this novel area of research has been hindered by an inability to uncover the molecular mechanisms regulating this behaviour. This study describes the TGF- β -ALK1-Smad1/5 signalling pathway as a key molecular mechanism regulating vasculogenic mimicry behaviour. We report that TGF- β signalling is expanded in some glioblastoma tumour cells allowing for TGF- β mediated activation of Smad1/5 signalling, which regulates tumour cell trans-differentiation into an endothelialised phenotype. Significantly, through discovery of this molecular mechanism we have been able to establish a novel mode of tumour dissemination contributing to wide-spread brain infiltration in glioblastoma (Figure 5.13). This finding is consistent with the widespread glioblastoma infiltration reported in the early radical surgical interventions performed by Dr Walter Dandy in the 1920s and more recently with immunohistochemical IDH1 mutant staining of secondary glioblastoma demonstrating the presence of single tumour cells distal from the primary tumour.^{222,429} Significantly, TGF- β signalling activity has been found to be upregulated in the perivascular niches and associated closely with stemness markers such as Id1, CD44, Sox4 and Sox2, suggesting an underlying inherent plasticity of these cells.^{374,376-378} Inhibition of TGF- β in these niches has been shown to prevent tumour recurrence development in intracranial mouse models, supporting our own findings.³⁷⁷

These findings, however, should be considered in light of recent glioblastoma genomic studies demonstrating that the major drivers for tumour progression are present at the time of diagnosis.³⁶ Given this tumourigenic evolution for glioblastoma, it is likely that tumour

endothelialisation and whole brain dissemination may have already occurred by the time of diagnosis. Future research endeavours should consider early detection and targeting of glioblastoma to provide the optimal opportunity for therapeutic success.

While glioblastoma has been reported to present with the highest number of vasculogenic mimicry cases, other aggressive cancers such as breast cancer and melanoma have also reported the presence of vasculogenic mimicry.³⁴⁴ Experimental evidence in our lab also suggests that the TGF- β -Smad1/5 pathway regulates vasculogenic mimicry in these cancers, providing a ubiquitous and comprehensive mechanism for this phenomenon that provide therapeutic targets in a range of solid malignancies (data not shown).

These results present a new biology for glioblastoma dissemination and probable recurrence, in addition, to establishing a functional role for vasculogenic mimicry. While previous studies have described an association between vasculogenic mimicry and late-stage/aggressive cancer progression and metastasis, this is the first time that vasculogenic mimicry has been described as actively participating in tumour dissemination through hematogenous routes.^{26,296-300}

Although our study has provided evidence of a novel biological pathway for tumour infiltration, it is important to consider that the MU20 cells used in this study also presented with extensive systemic brain infiltration, though these cells demonstrated a remarkable capacity for cellular proliferation and invasion in our mouse model. This mode of tumour dissemination is consistent with well characterised neuropil invasion, which is currently considered as the only relevant mode of infiltration.⁴⁵⁰ Further research will need to be conducted to determine the overall contribution of these modes of infiltration and their implications for glioblastoma development and progression.

Our study also demonstrated the anti-tumourigenic effects of Smad7, inhibiting both Smad2/3 and Smad1/5 pathways. Ikushima *et al.*, has reported a similar finding with SB431542 pre-treatment of glioblastoma-initiating cells leading to complete abolishment of tumourigenic capacity in intracranial mouse models.³⁷⁷ Significant in both our study designs, is early treatment during initial tumour development. Our study differs, however, in the unique intracranial route for treatment, allowing for treatment of not only the tumour but also the surrounding microenvironment. Other studies have also reported a role for TGF- β signalling in maintenance of a stem-cell-like phenotype and tumourigenesis *in vivo*.^{374,376} These results present a promising direction for therapeutic targeting of the TGF- β signalling pathway.

5.6 Conclusions

This study has identified tumour endothelialisation as a novel tumour biology leading to systemic brain tumour infiltration. These findings provide a new explanation for the aggressive and incurable nature of glioblastoma and may be a promising therapeutic target to combat tumour recurrence.

Figure Legends

Figure 5.1. Some glioblastoma tumour cell lines seeded onto Matrigel mimic endothelial-like lattice formation structures *in vitro*.

A, Glioblastoma cell lines were seeded onto empty (control) or matrigel coated well in a 48-well tissue culture plate, which was incubated at 37°C. Images of control and Matrigel coated wells were captured following 6hrs of incubation using a Leica fluorescence microscope at X100 total magnification.

B, Representation of angiogenesis quantitation from U118MG Matrigel seeded image (above). Quantitation was performed using the angiogenesis analyser addon with ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>).

C, Quantitation of lattice formation structures, n=3 for each cell line. Data is mean $\pm$ SEM. *** $P < 0.001$, **** $P < 0.0001$; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.2. TGF- β -Smad1/5 signalling is expanded in some glioblastoma tumour cell lines.

A, U87MG glioblastoma cells were concomitantly transduced with 250 MOI BRE-FLUC and 100 MOI CAGA-GLUC. Cells were serum starved and treated with various concentrations of TGF- β for 6hrs. Cells were lysed, and luciferase activity measured.

B, U87MG were serum starved and treated with various concentrations of TGF- β . Cells were lysed and 0, 0.5, 1 and 2hrs and probed for protein expression via western blot analysis (left panel). Quantitation of western blot normalised to actin for pSmad1/5 (middle panel) and pSmad2/3 (right panel).

C, Glioblastoma cells were concomitantly transduced with 250 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC. Cells were serum starved and treated with 2ng/mL TGF- β or 20ng/mL BMP7 for 6hrs. Cells were lysed, and luciferase activity measured. n=3 for each cell line. Data is mean $\pm$ SEM. **P<0.01, *** P<0.001, **** P<0.0001; One-way ANOVA with Tukey's multiple comparisons test.

D, Glioblastoma cells were serum starved and treated with 2ng/mL TGF- β or 20ng/mL BMP7 for 1hr, following which cells were lysed and probed for protein expression via western blot analysis (Top panel). Quantitation of western blot normalised to actin for pSmad1/5 (bottom left panel) and pSmad2/3 (bottom right panel).

E, Glioblastoma cell lines were transduced with 250 MOI BRE-FLUC and seeded into control or Matrigel coated wells for 6hrs. Cells were lysed, and luciferase activity measured. n=3 for each cell line. Data is mean $\pm$ SEM. *** P<0.001, **** P<0.0001; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.3. Ad-Smad6 and Ad-Smad7 reduces phosphorylation levels of Smad1 and can be successfully expressed in every cell in U87MG cells.

A, U87MG cells were transduced with 1000 MOI Ad-CMV-Tom (control) or 500, 1000 and 2000 MOI Ad-Smad6-M2 or Ad-Smad7-M2. 48hrs later cells were lysed and protein expression probed via western blot analysis.

B, U87MG cells were transduced with 500 MOI Ad-CMV-Tom (control), Ad-Smad6-M2 or Ad-Smad7-M2. 48hrs later cells were fixed using 3.7% paraformaldehyde and permeabilised using 0.1% Triton-X. Following blocking in normal serum, cells were probed with primary anti-M2 antibody (1:1000 dilution) for 30mins and secondary ALEXA-546 antibody (1:5000

dilution) for 1hr. Images were captured using a Leica Fluorescent Microscope X200 total magnification.

C-F, Glioblastoma cell lines were concomitantly transduced with 250 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC seeded into a 96 well plate at a cell density of 5×10^3 . Cells were serum starved and treated with 2ng/mL TGF- β or 20ng/mL BMP7 for 6hrs. Cells were then lysed, and luciferase activity measured. U87MG (A), U118MG (B), MU20 (C) and MU41 (D). BRE-FLUC – left panels, CAGA-GLUC – right panels. n=3 independent experiments for each cell line. Data is mean $\pm$ SEM. *** $P < 0.001$, **** $P < 0.0001$; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.4. Smad6 overexpression attenuates lattice formation in glioblastoma cell lines.

A, Glioblastoma cell lines were transduced with Ad-CMV-Tom (control), Ad-Smad6 or Ad-Smad7 and incubated at 37°C for 48hrs. Cells were then seeded onto uncoated control or matrigel coated wells in a 48-well tissue culture plate and incubated at 37°C for 6hrs. Images of lattice formation were captured using a Leica fluorescence microscope at X100 total magnification. Empty controls are not presented.

B, Lattice formation images were quantified using the angiogenesis analyser addon with ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>). n=3 independent experiments for each cell line. Data is mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to control; One-way ANOVA with Tukey's multiple comparisons test. Meshes and junctions were not detected with MU20 cells and no graphs are presented for these variables for this cell line.

C, Following image acquisition of lattice formation, both uncoated control and Matrigel coated wells were lysed and BRE-FLUC luciferase activity was measured. U87MG (top left

panel), U118MG (top right panel), MU20 (bottom left panel), MU41 (bottom right panel).

n=3 independent experiments. Data is mean $\pm$ SEM. **** P<0.0001; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.5. SB-431542, a T β RI inhibitor, completely abolishes lattice formation behaviour in U87MG cells.

A, U87MG cells were concomitantly transduced with 250 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC and seeded at a density of 5×10^3 cells in a 96 well plate. Cells were serum starved and treated with 2ng/mL TGF- β along with either DMSO (control) or 10 μ M SB-431542 for 6hrs. Cells were lysed, and luciferase activity measured.

B, U87MG cells were seeded onto Matrigel coated wells of a 96 well plate at a cell density of 1×10^4 and simultaneously treated with DMSO (control) or 10 μ M SB-431542. Images were captured using a Leica fluorescent microscope.

C, Image captured in (B) were quantified using the angiogenesis analyser addon with ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>). n=3 independent experiments. Data is mean $\pm$ SEM. ****P<0.0001; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.6. Knockdown of ALK1 receptor inhibits lattice formation in U87MG cells.

A, U87MG were stably transfected with vector or ALK1-shRNA 354, 355 or 356. Stable clones were generated, and successful knockdown was confirmed via western blot (top panel). Quantitation of western blot normalised to actin for ALK1 expression (bottom panel). Western blot is representative of 3 individual experiments.

B, U87MG clones were concomitantly transduced with 250 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC and seeded at a density of 5×10^3 cells in a 96 well plate. Cells were

serum starved and treated with 2ng/mL TGF- β along for 6hrs. Cells were lysed, and luciferase activity measured. BRE-FLUC activity – top panel, CAGA-GLUC – bottom panel. n=3 independent experiments. Data is mean $\pm$ SEM. **P<0.01, ***P<0.001; One-way ANOVA with Tukey's multiple comparisons test.

C, U87MG clones were transduced with 250 MOI Ad-BRE-FLUC and incubated for 48hrs at 37°C. Cells were then seeded onto uncoated or coated Matrigel wells in a 96 well plate at a cell density of 1×10^4 for 6hrs. Images were captured using a Leica fluorescent microscope. Representative images of 3 independent experiments. Cells seeded onto uncoated wells are not shown.

D, Image captured in (C) were quantified using the angiogenesis analyser addon with ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>). n=3 independent experiments. Data is mean $\pm$ SEM. *P<0.05, **P<0.01; One-way ANOVA with Tukey's multiple comparisons test.

E, Following image acquisition of lattice formation in (C), both uncoated control and Matrigel coated wells were lysed and BRE-FLUC luciferase activity was measured.

Figure 5.7. Overexpression of a constitutively active ALK1 receptor activates BRE transcription and results in lattice formation in the TGF- β signalling deficient cell line MU20.

A, MU20 cells were transduced with 500 MOI Ad-caALK1 or Ad-CMV-Tom. Expression of ALK1 receptor was confirmed via western blot (top panel). Next MU20 cells were concomitantly transduced with 250 MOI BRE-FLUC and 100 MOI CAGA-GLUC. Cells were incubated for 48hrs at 37°C, following which they were lysed and luciferase activity measured. BRE-FLUC activity – bottom left panel. CAGA-GLUC activity – bottom right

panel. n=3 independent experiments. Data is mean $\pm$ SEM. ****P<0.0001; unpaired student's t test.

B, MU20 cells were transduced with Ad-CMV-Tom (control) or Ad-caALK1 and incubated at 37°C for 48hrs. Cells were then seeded on Matrigel coated wells of a 96 well plate at a density of 1.5×10^4 and incubated at 37°C. Images were captured 24hrs later using a Leica fluorescent microscope (x100 total magnification). Image are representative of 3 independent experiments.

C, Image captured in (B) were quantified using the angiogenesis analyser addon with ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>). n=3 independent experiments. Data is mean $\pm$ SEM. *P<0.05, **P<0.01; unpaired student's t test.

Figure 5.8. U87MG but not MU20 glioblastoma cells incorporate into the tumour vasculature *in vivo*.

A, 5×10^4 U87MG-FLUC and MU20-FLUC were implanted into the right cortex of NOD-SCID mice. Bioluminescence images were captured at day 13, 19 and 26 post-implantation using the Caliper IVIS Lumina II system. Representative images of bioluminescence signal from U87MG-FLUC and MU20-FLUC tumours (left panel). Quantitation of total flux for U87MG-FLUC and MU20-FLUC tumours (right panel), n=6 for each group. Data is mean $\pm$ SEM.

B, Body weights of mice implanted with either U87MG-FLUC or MU20-FLUC tumours over the duration of the experiment. Data is mean $\pm$ SEM.

C, Representative immunohistochemistry H&E staining of U87MG-FLUC and MU20-FLUC tumours. Arrows indicate clustering of invasive cells moving beyond the primary tumour margin.

D-E, Representative immunofluorescent images of U87MG-F-Luc (D) and MU20-F-Luc (E) tumour sections. Arrows indicate CD31-/HLA-A⁺ vasculature staining (bottom panels). A section of tumour vasculature was expanded and the intensity of CD31⁺ and HLA-A⁺ staining across the circumference of the tumour vasculature was measured (bottom right panels). Numbers refer to the spatial positioning on the circumference of the tumour vasculature.

F, Quantitation of immunofluorescence images. Endothelialised cells were defined as CD31⁺/HLA-A⁺ cells spatially incorporated into the tumour vasculature. An endothelialised vessel was considered any blood vessel with the presence of one or more endothelialised cells. n=4 for each group. Data is mean $\pm$ SEM. **P<0.01, ****P<0.0001; unpaired student's t test.

Figure 5.9. Intracranial Smad6 treatment inhibits glioblastoma endothelialisation *in vivo*.

A, Schematic diagram of the orthotopic contralateral xenograft mouse model. 2×10^5 U87MG-GLuc cells were transduced with 500 MOI Ad-BRE-FLuc and 48hrs later were stereotactically implanted into the right hemisphere of the mouse brain. 4 days later 2×10^5 U87MG wild-type cells were implanted into the contralateral hemisphere. IVIS bioluminescence imaging was performed on day 9 followed directly by intracranial treatment with 10 μ L Ad-CMV-TOM, Ad-Smad6 or AD-Smad7 adenovirus. IVIS imaging was again performed on day 11 and all mice were euthanised at experimental end-point at day 16.

B, Body weights of mice by treatment group. Day 1 corresponds to experimental start date with tumour implantation. Data is mean $\pm$ SEM.

C, IVIS bioluminescence imaging capture before intracranial treatment (left panel) and 2 days after (right panel). n=6 for each group. Data is mean $\pm$ SEM. *P<0.05; unpaired student's t test.

D, Representative immunohistochemistry H&E staining of U87MG-FLUC tumours by treatment group. Smad7 treated tumours could not be observed at experimental end-point and images for these group were not included.

E-F, Representative immunofluorescent images of control (E) and Smad6 (F) treated tumour sections. Arrows indicate CD31-/HLA-A+ vasculature staining (bottom panels).

G, Quantitation of immunofluorescence images. Endothelialised cells were defined as CD31-/HLA-A+ cells spatially incorporated into the tumour vasculature. An endothelialised vessel was considered any blood vessel with the presence of one or more endothelialised cells. n=4 for each group. Data is mean $\pm$ SEM. *P<0.05; unpaired student's t test.

Figure 5.10. Intracranial Smad6 inhibition reduces whole brain tumour dissemination, while Smad7 treatment abolishes primary tumour growth

A-C, U87MG-G-Luc labelled/unlabelled tumours and peripheral brain samples were harvested following euthanasia. Samples were homogenised and 10mg tissue was used to measure luciferase activity. n=5 mice for Ad-CMV-Tom group, n=6 for Ad-Smad6 and Ad-Smad7 groups. In the labelled tumour samples, dual-luciferase analysis was performed, first measuring BRE-F-Luc activity (A; right panel), followed by CMV-G-Luc activity (A; left panel). CMV-G-Luc activity were also measured in the unlabelled tumour (B; left panel) and peripheral brain (C; left panel). For peripheral brain measurements, 3 independent brain samples are presented for each mouse. Relative luciferase activity in the unlabelled tumour samples and peripheral brain samples were extrapolated to total cell number using standard

curves (C-D; right panels). Data is mean $\pm$ SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; One-way ANOVA with Tukey's multiple comparisons test.

Figure 5.11. Glioblastoma tumour cells can incorporate into the endothelium of the tumour vasculature in humans.

In situ hybridisation (ISH) and H&E images were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP) (<https://glioblastoma.alleninstitute.org/>). Images are representative of a single patient with confirmed *FGFR3-TACC3* fusion gene.

A, A tumour blood vessel with *FGFR3-TACC3* positive ISH staining, arrows indicate positive staining within the endothelium (left panel). Pseudo-colouring of image shown in the left panel for *FGFR3-TACC3* positive cells (middle panel). Nearest H&E section (right panel).

B-D, A tumour blood vessel showing endothelial enriched ISH staining, arrows indicate negative staining within the endothelium (left panel). Pseudo-colouring of image shown in the left panel for endothelial enriched markers positive cells (middle panel). Nearest H&E section (right panel).

Figure 5.12. Glioblastoma patient samples exhibit non-continuous endothelial cell marker expression with non-endothelial cells incorporated into the endothelium of tumour vasculature.

In situ hybridisation (ISH) and H&E images were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP) (<https://glioblastoma.alleninstitute.org/>). Images are representative of multiple patients.

A-D, A tumour blood vessel showing endothelial enriched ISH staining, arrows indicate negative staining within the endothelium (left and middle panels). A tumour blood vessel showing continuous endothelial enriched ISH staining (right panels).

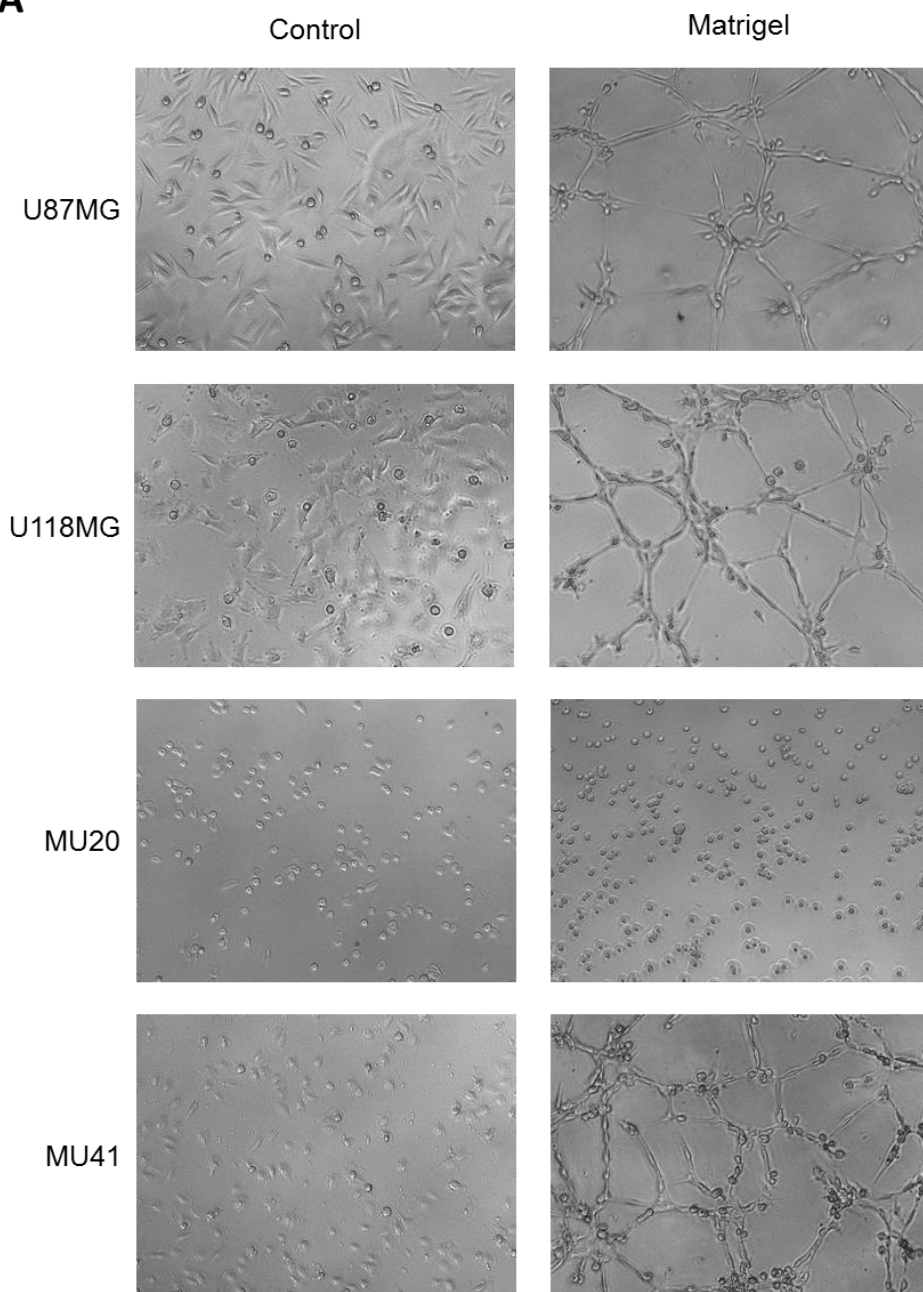
E, A tumour blood vessel with *GFAP* positive ISH staining, arrows indicate positive staining within the endothelium.

Figure 5.13. TGF- β -Smad1/5 induces tumour cell endothelialisation and distal brain tumour dissemination.

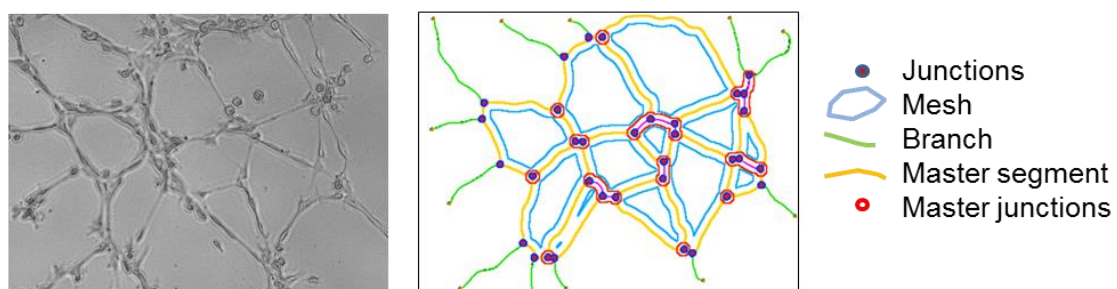
Figures

Figure 5.1

A



B



C

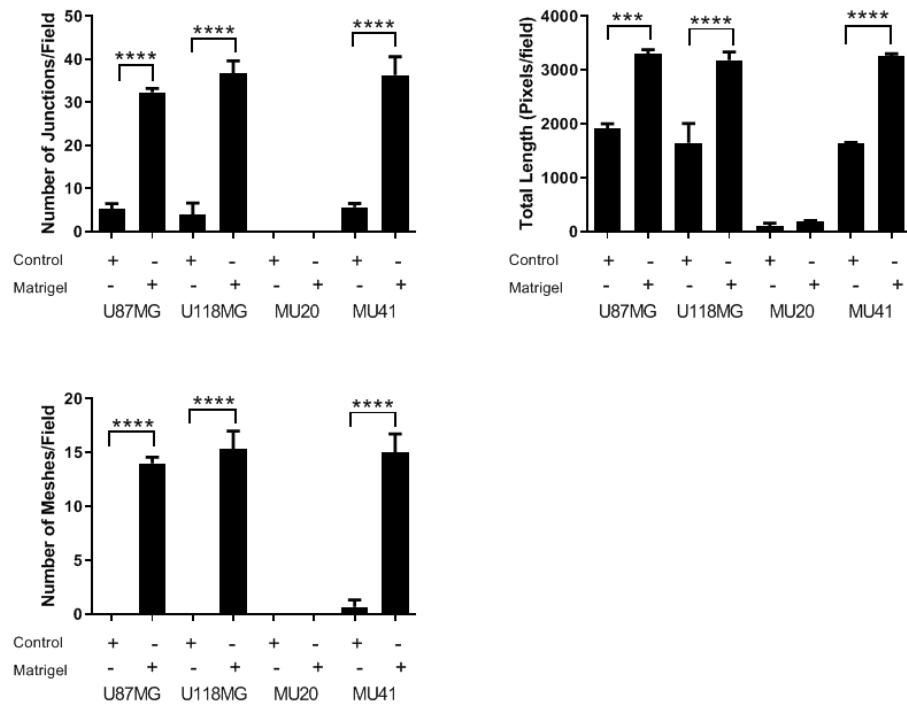


Figure 5.2

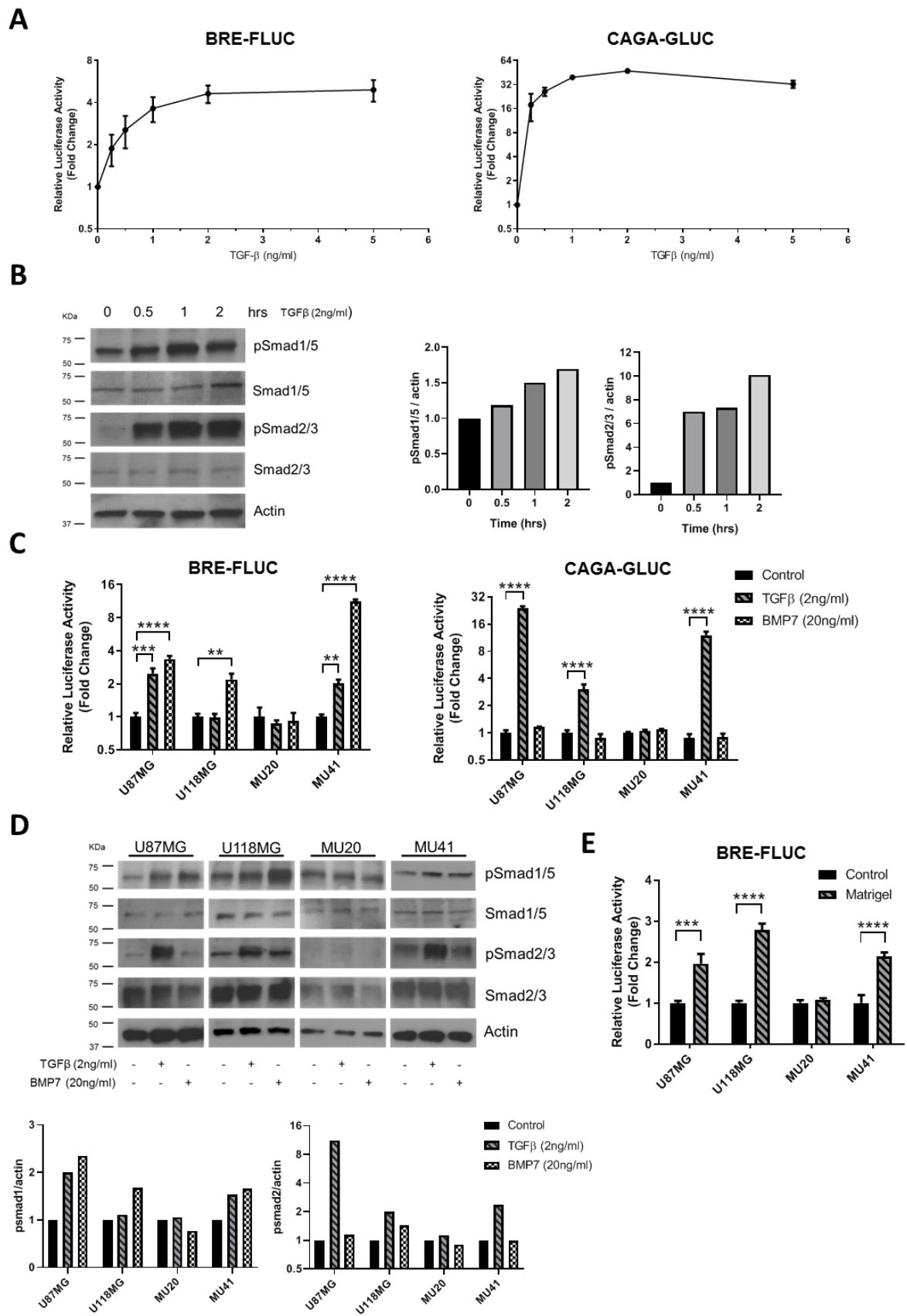
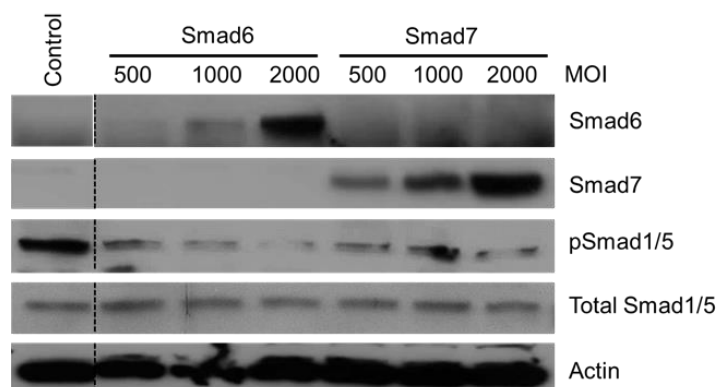


Figure 5.3

A



B

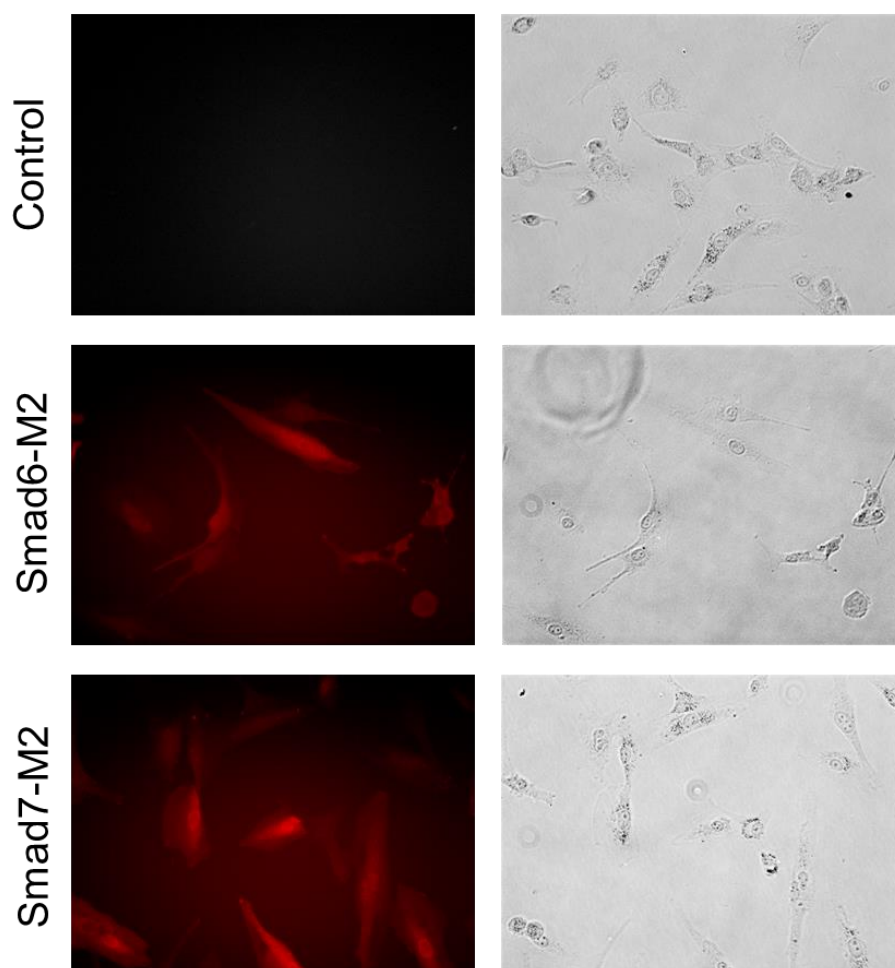
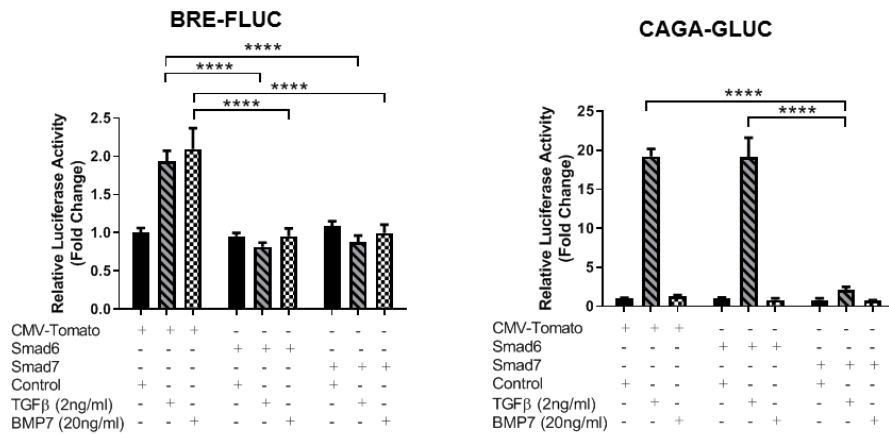
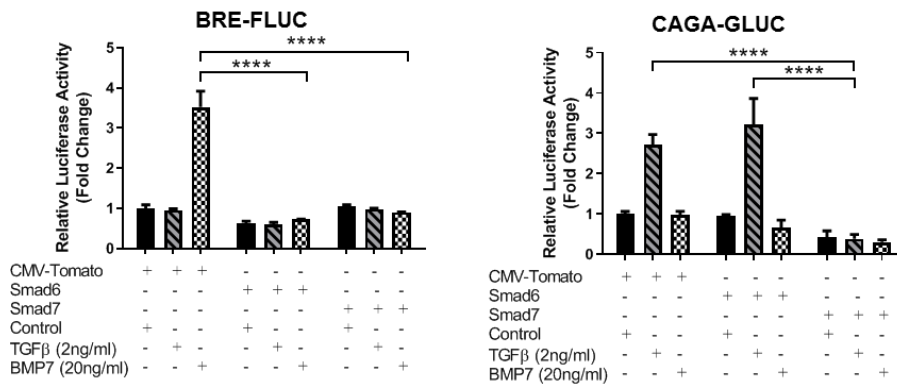


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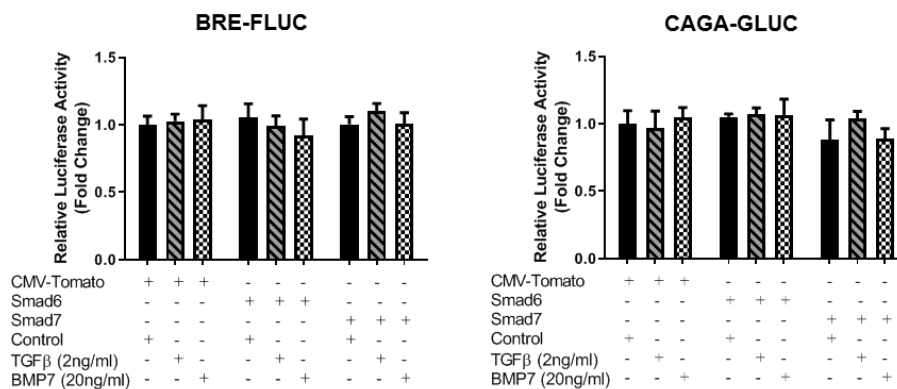
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D



E



F

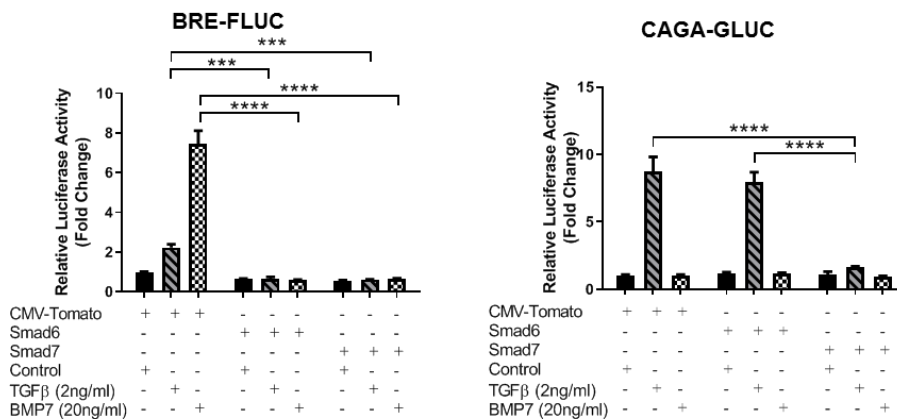


Figure 5.4

A

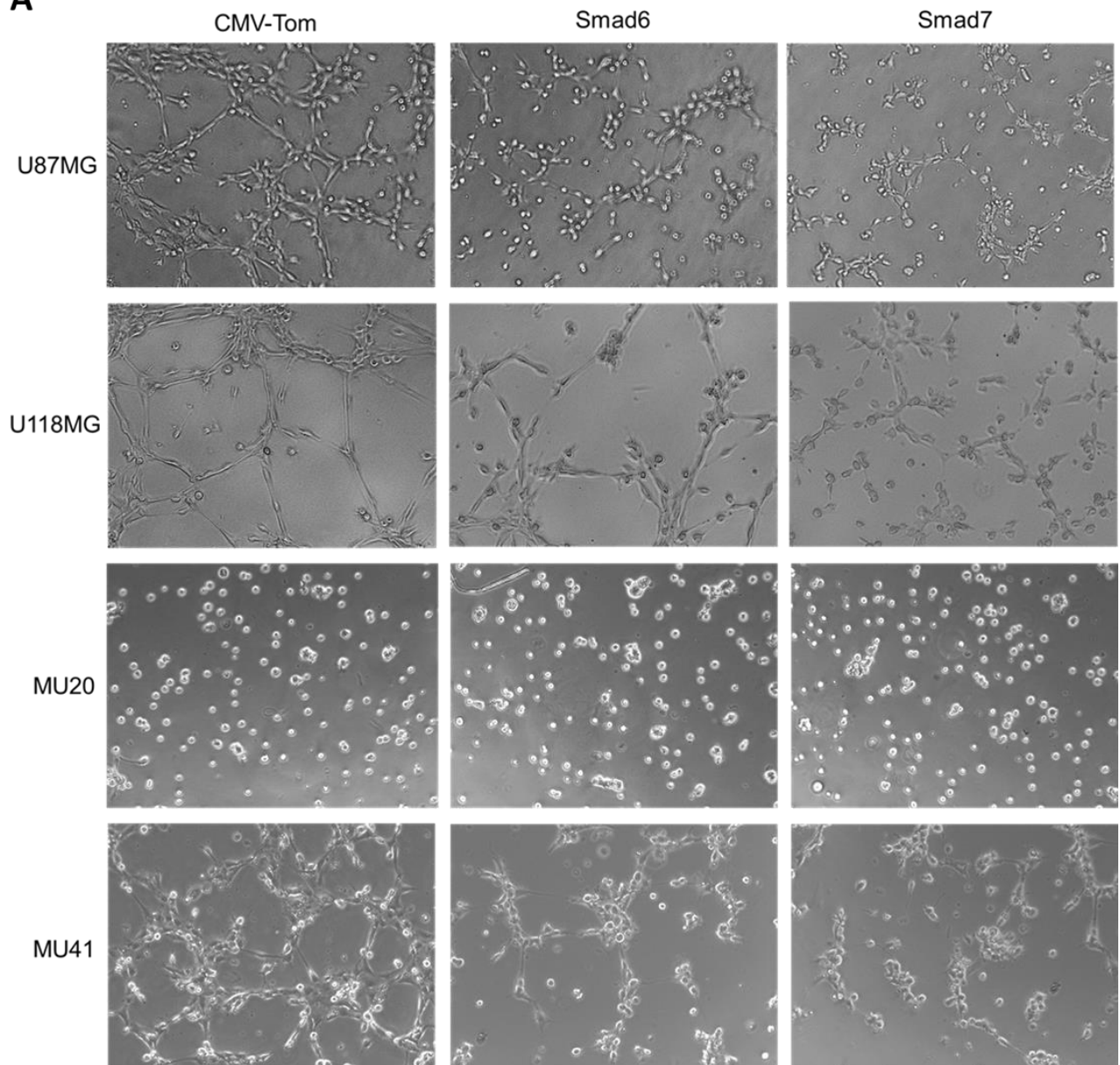


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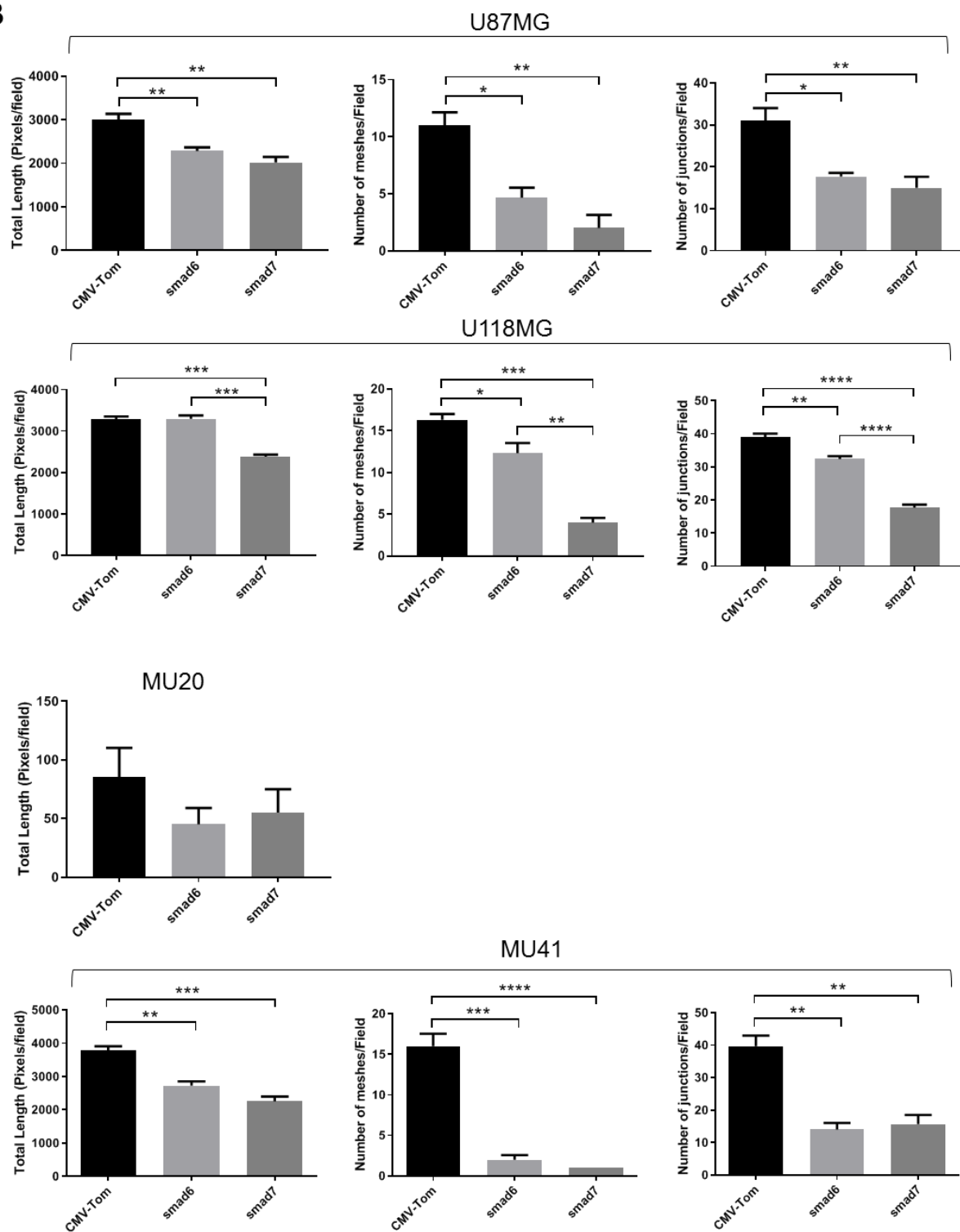


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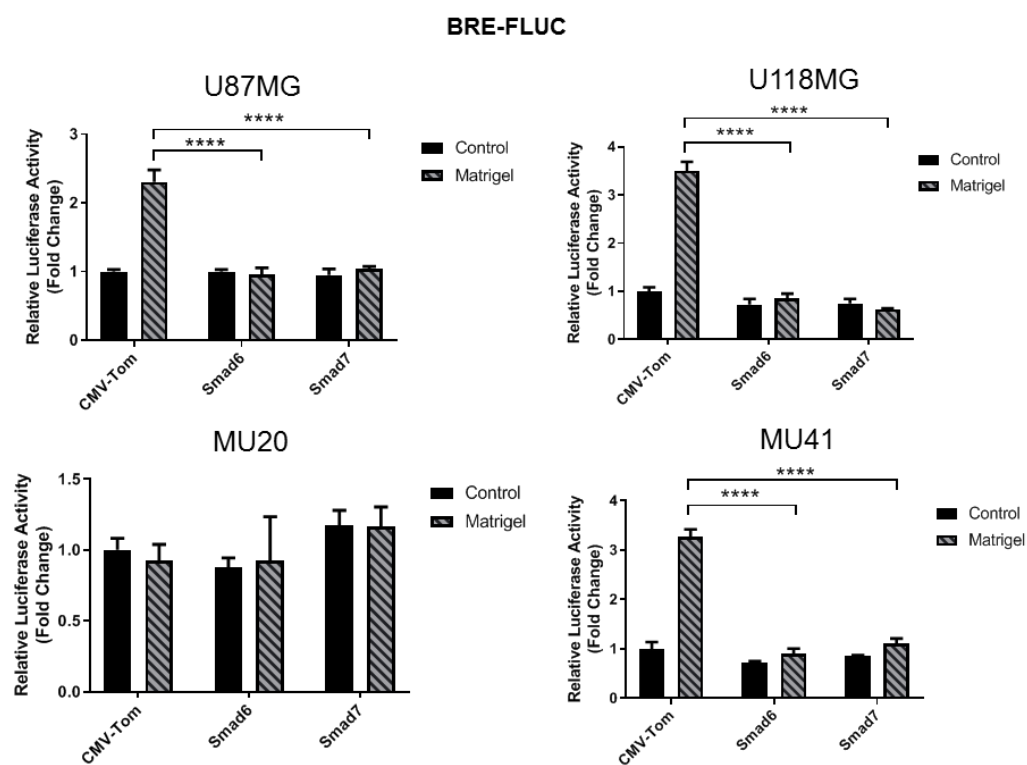


Figure 5.5

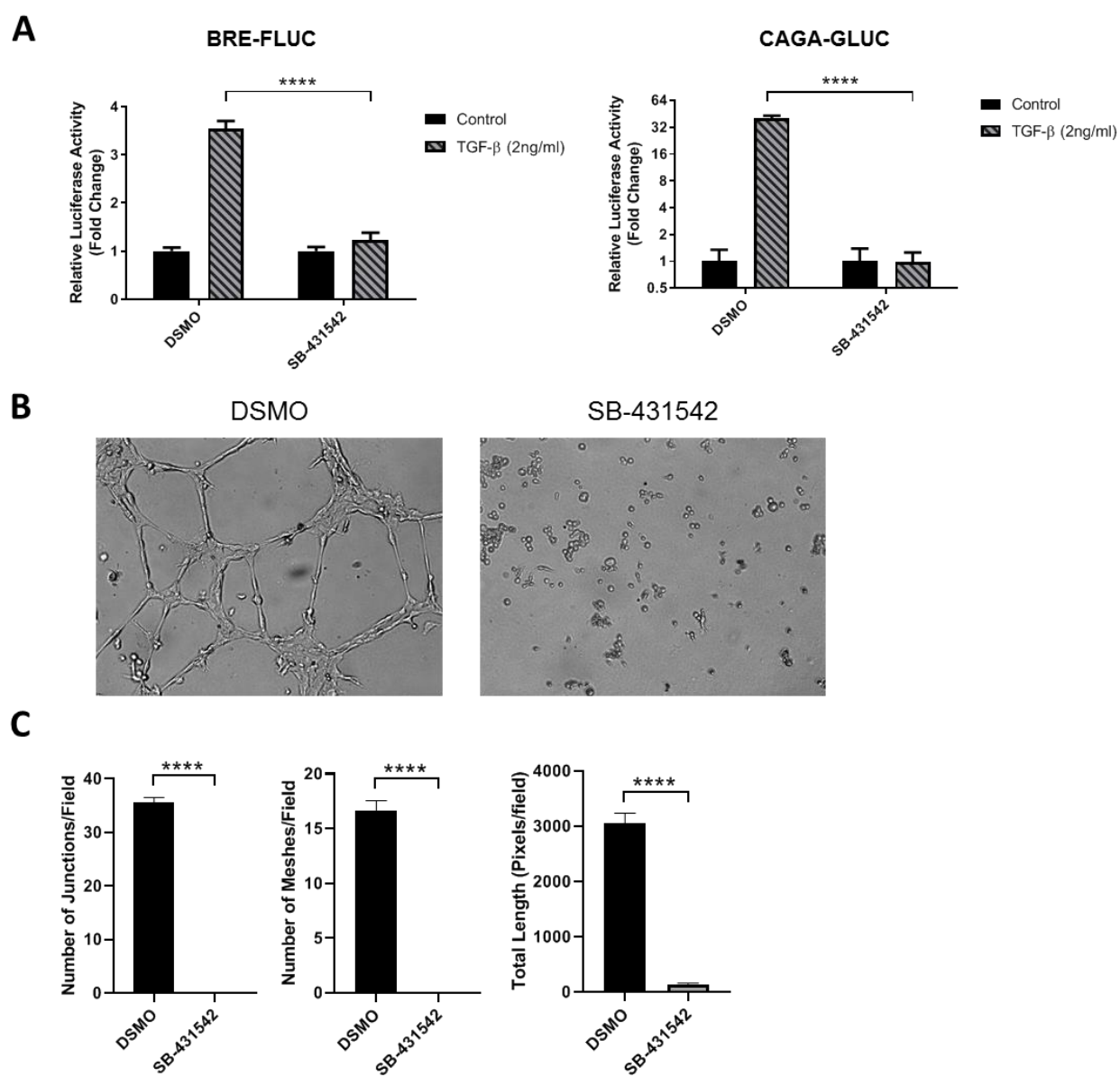


Figure 5.6

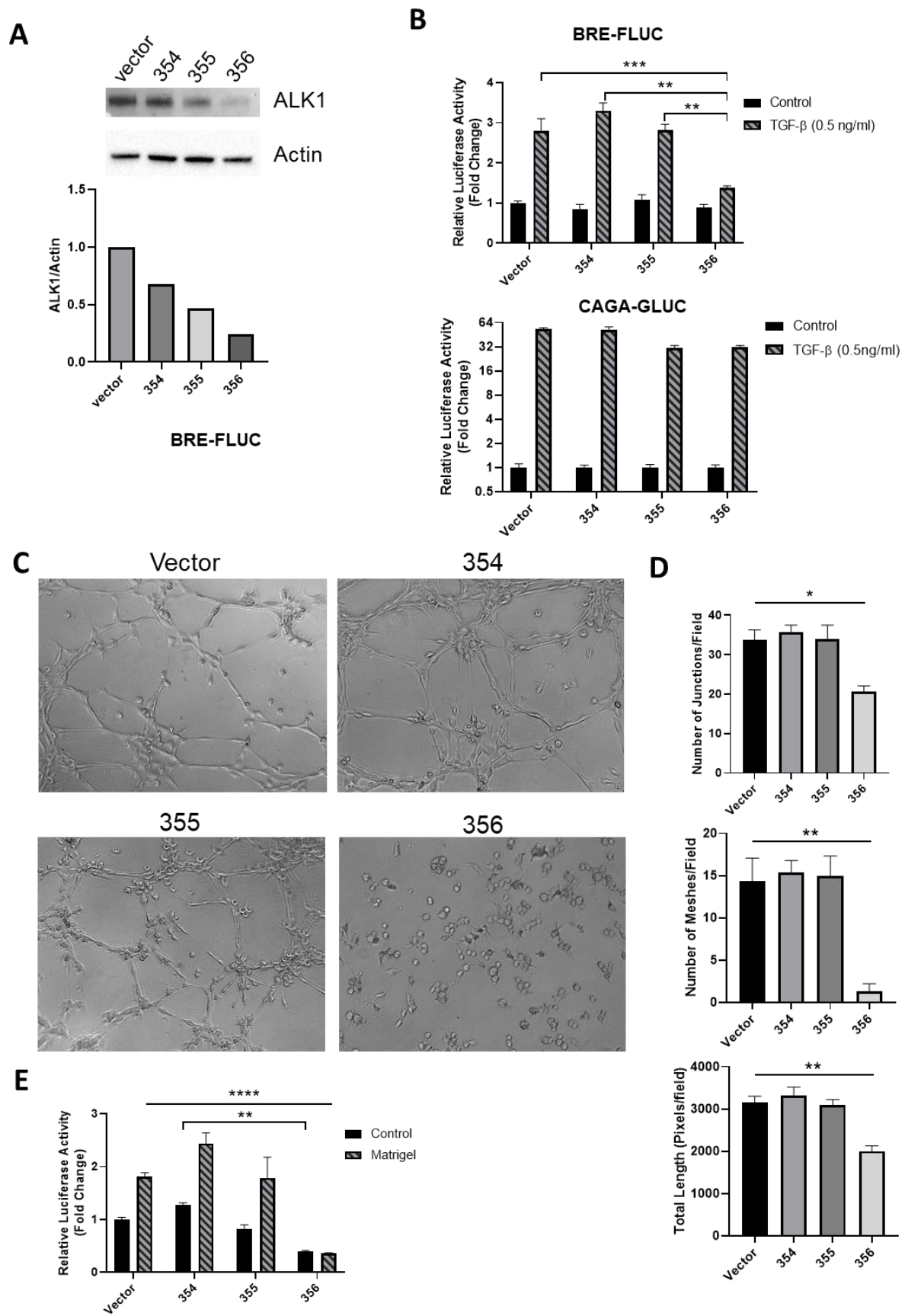
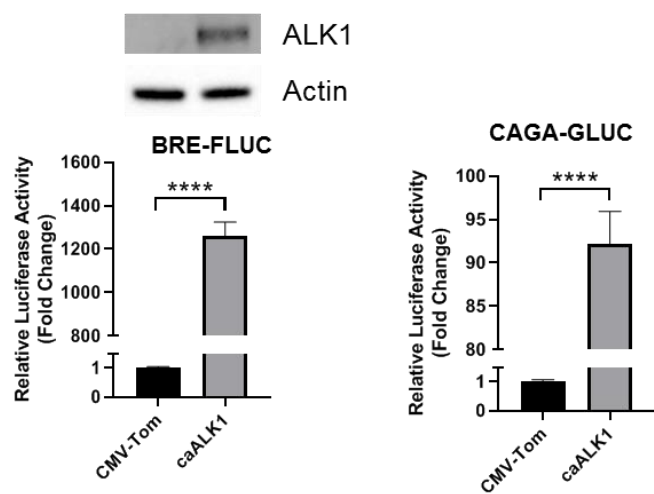
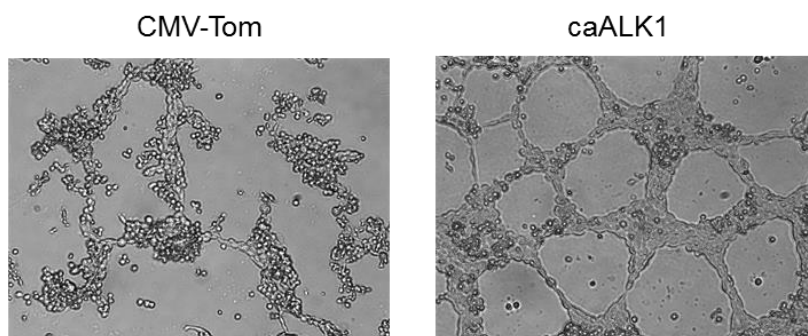


Figure 5.7

A



B



C

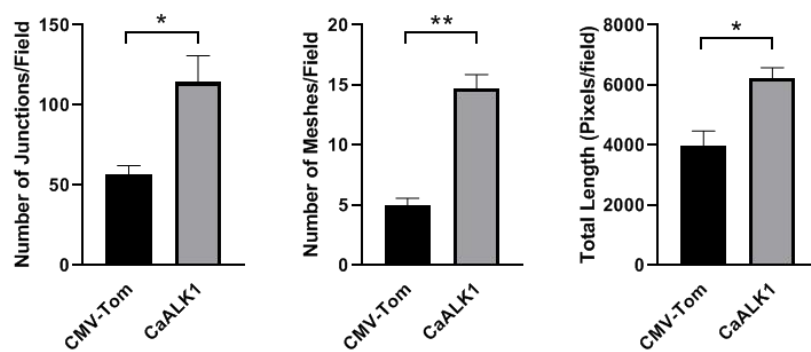
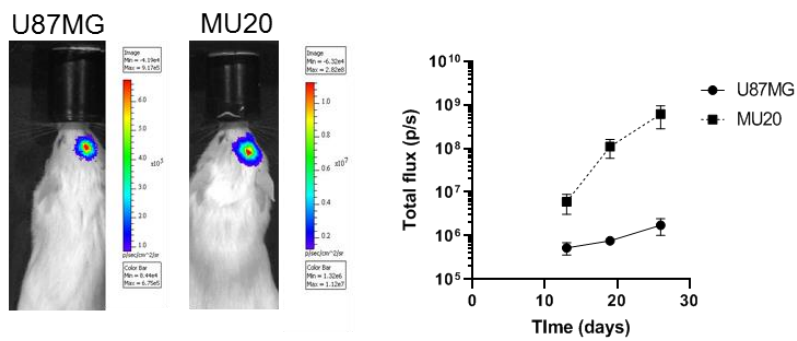
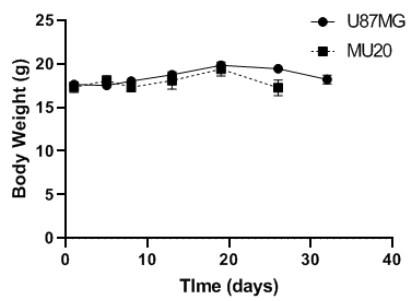


Figure 5.8

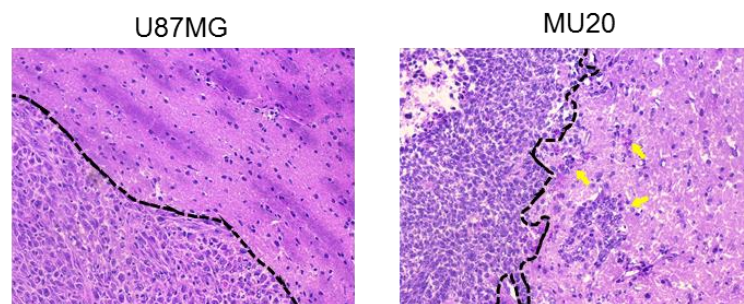
A



B



C



D

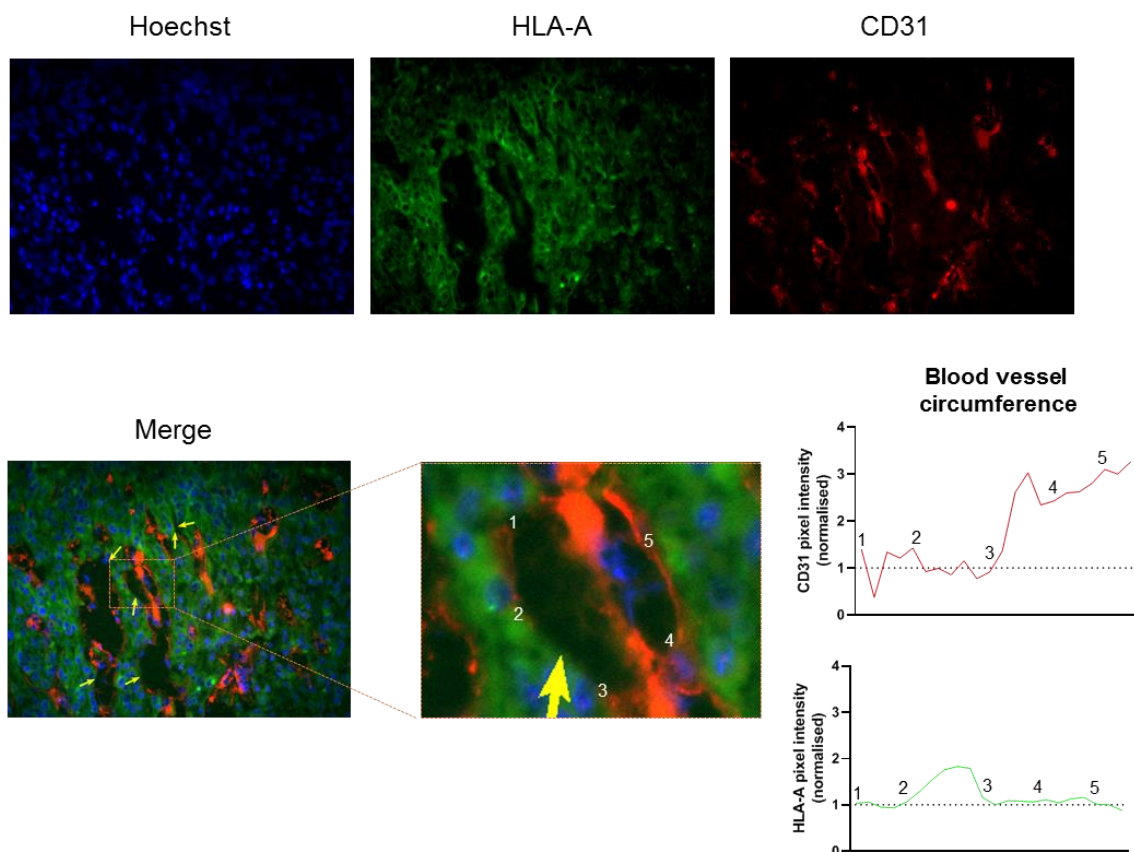
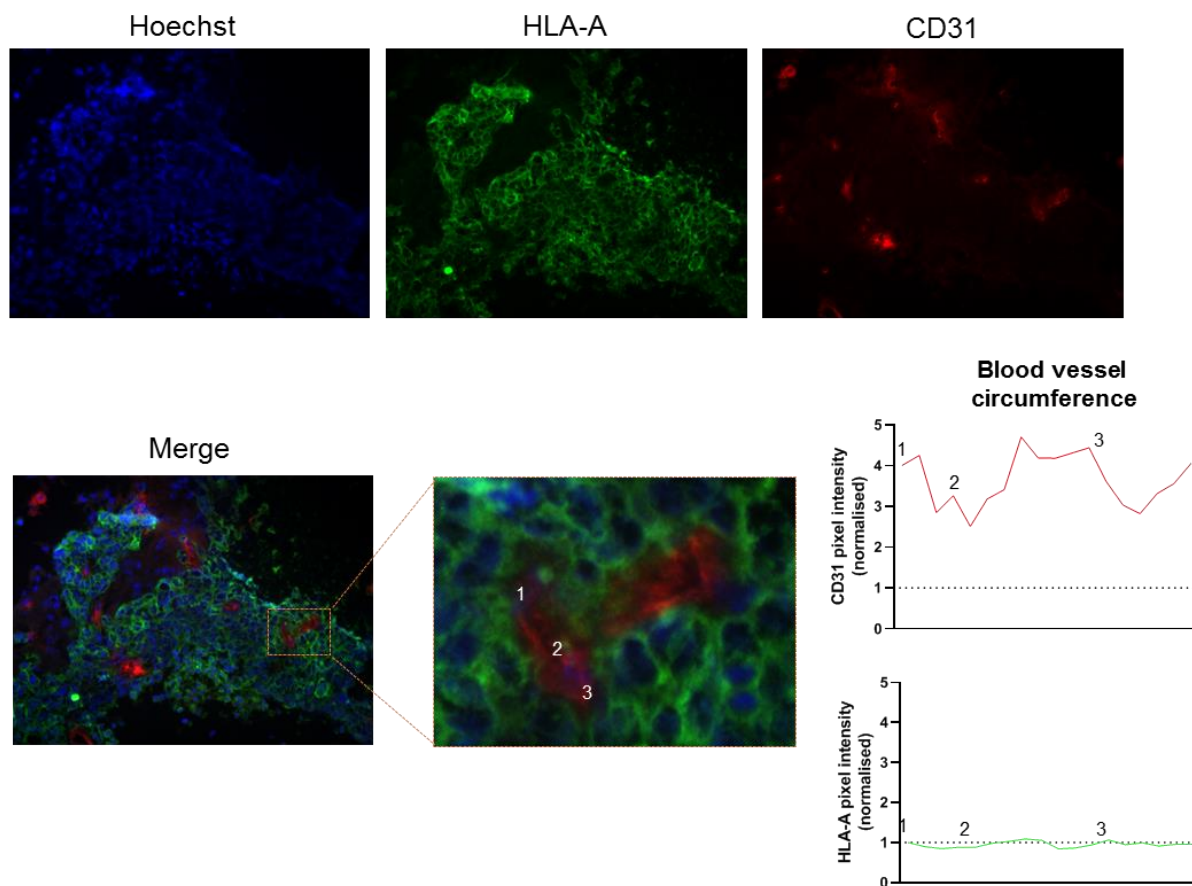


Figure 5.8 (continued)

E



F

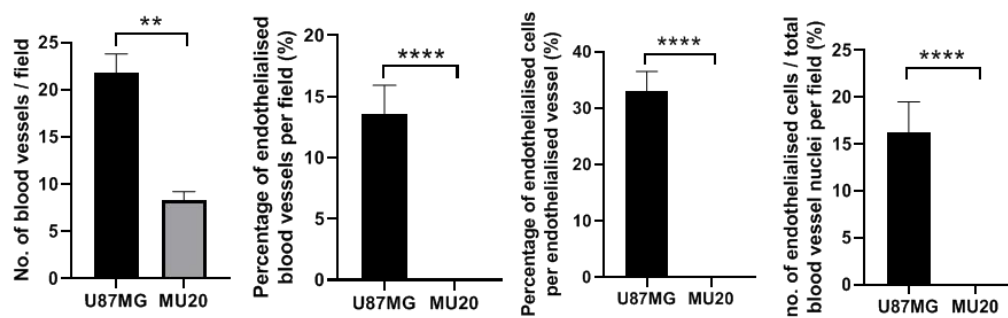


Figure 5.9

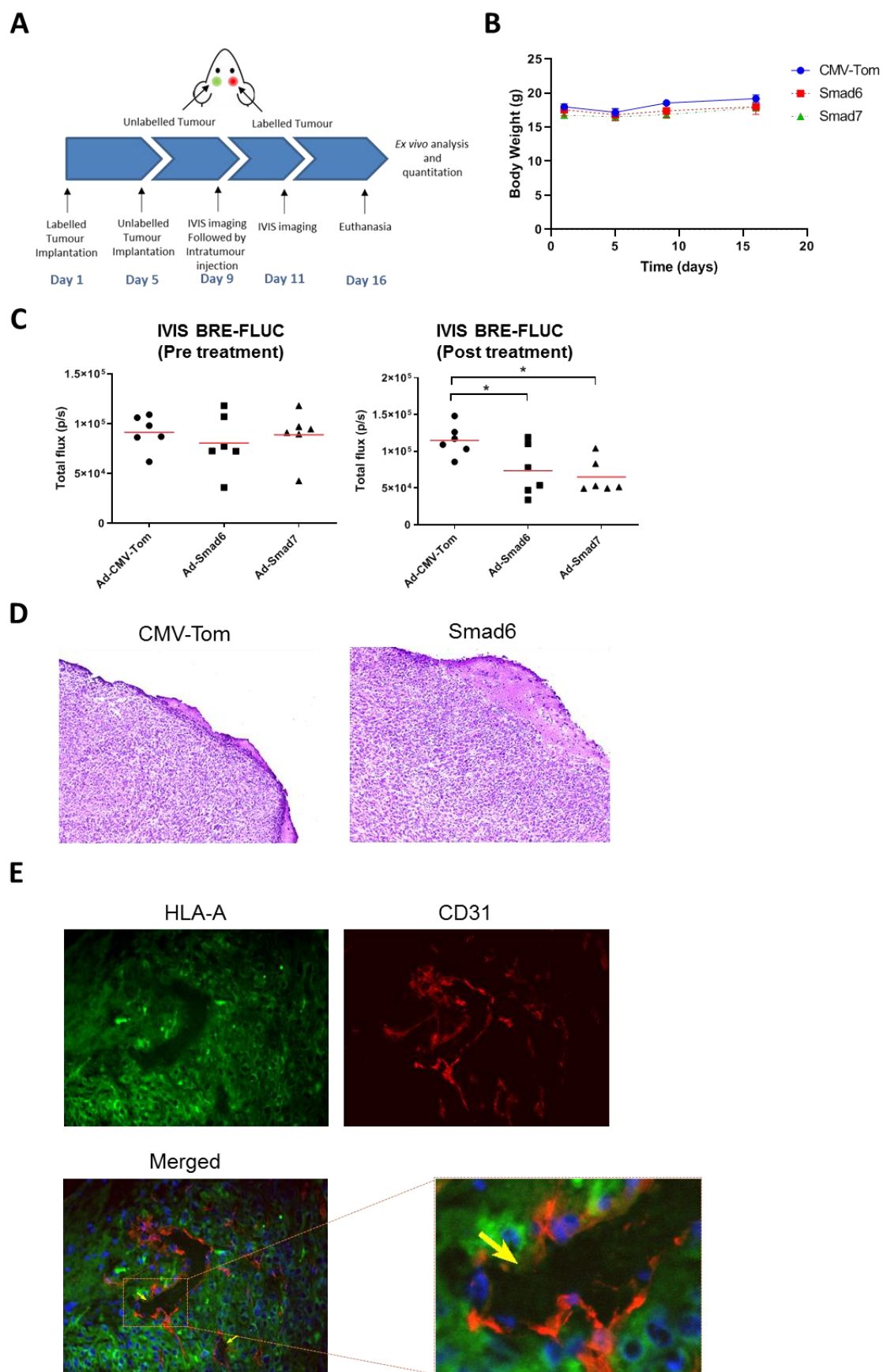
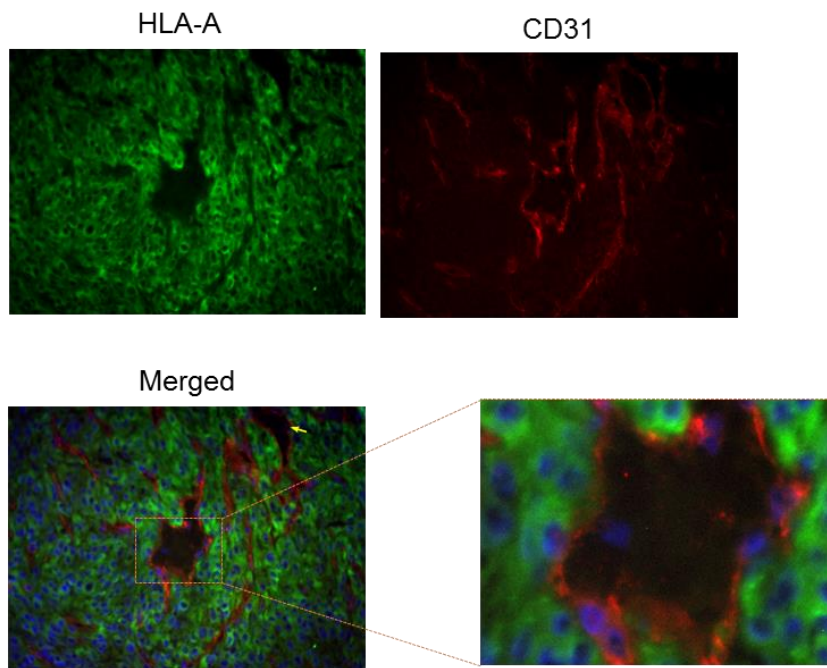


Figure 5.9 (continued)

F



G

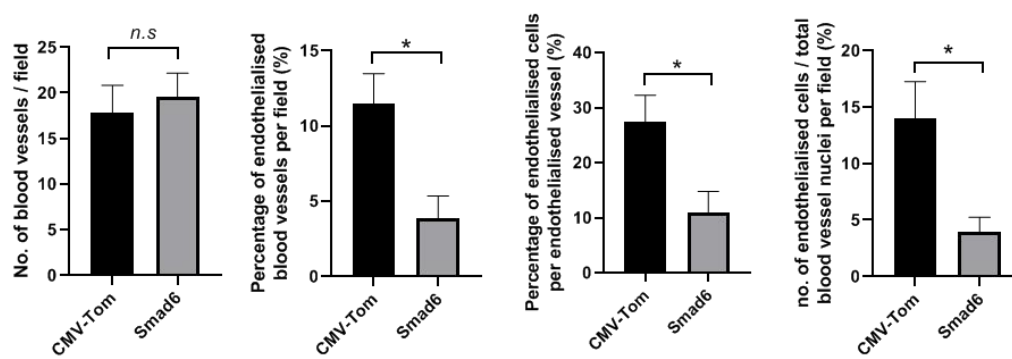
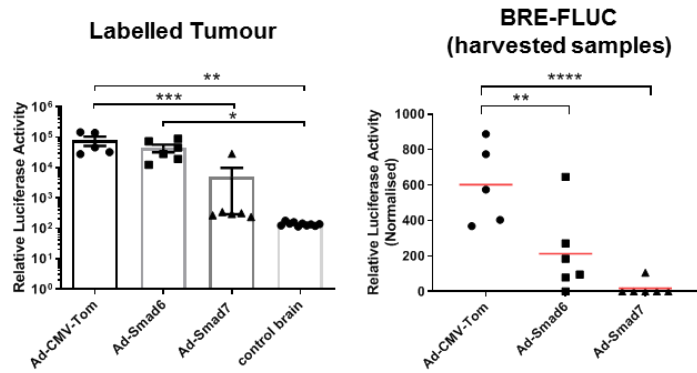
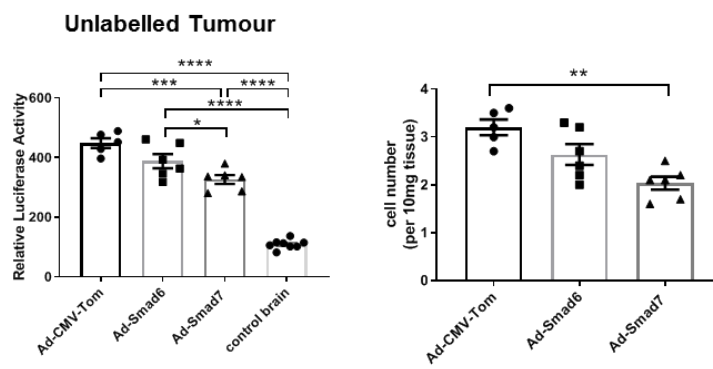


Figure 5.10

A



B



C

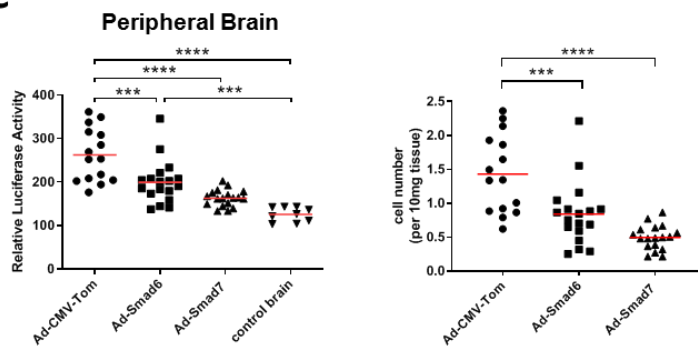


Figure 5.11

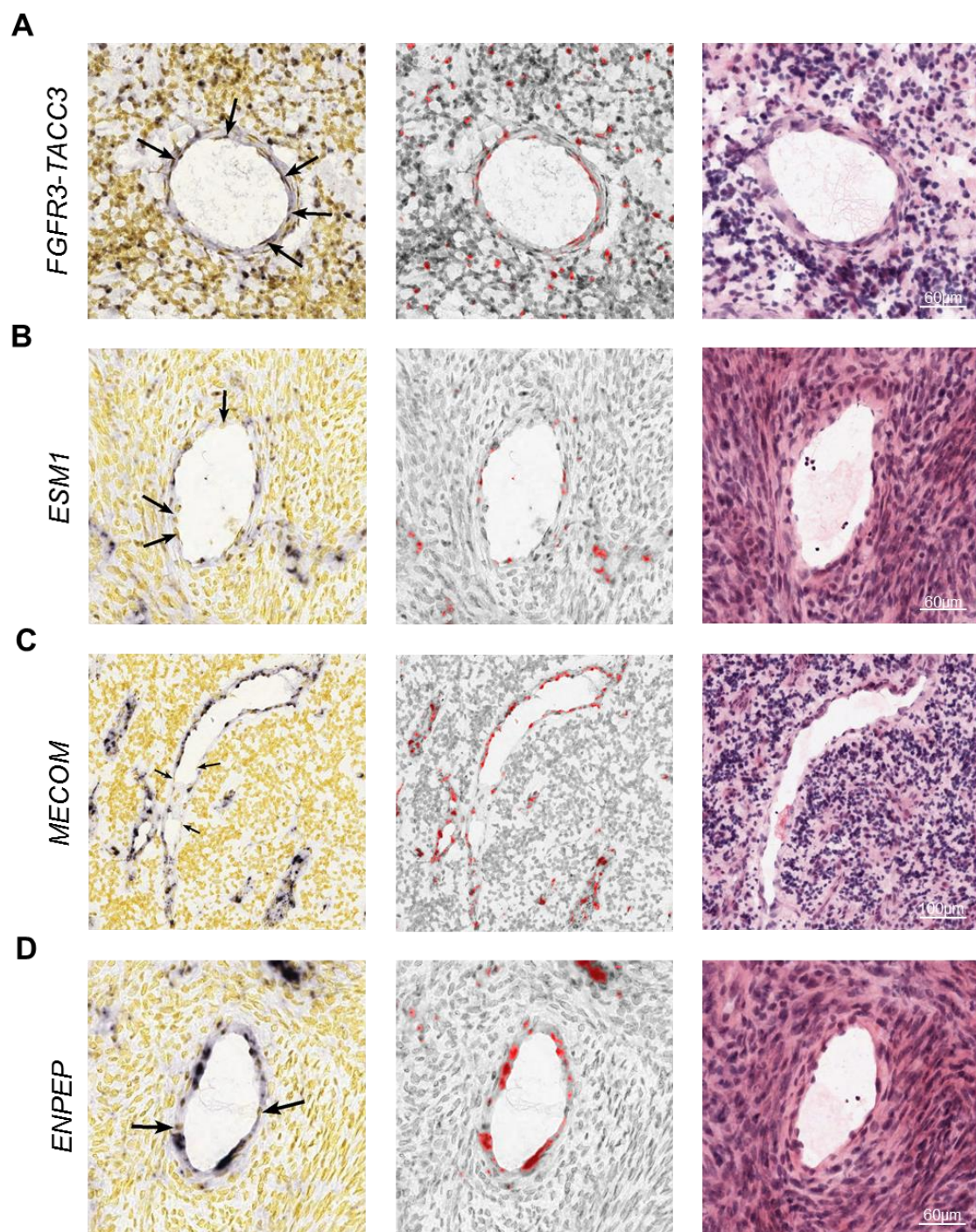


Figure 5.12

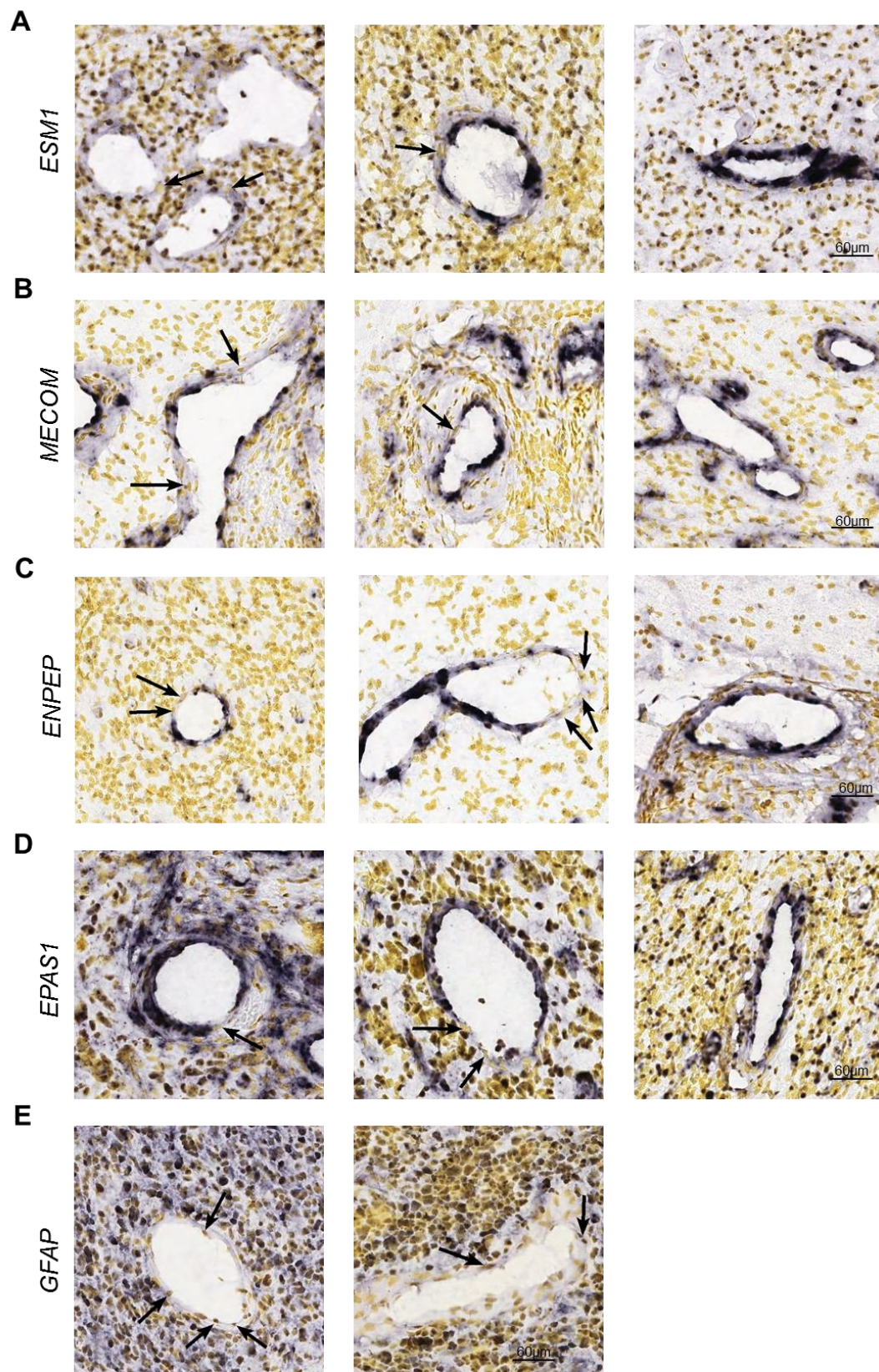


Figure 5.13

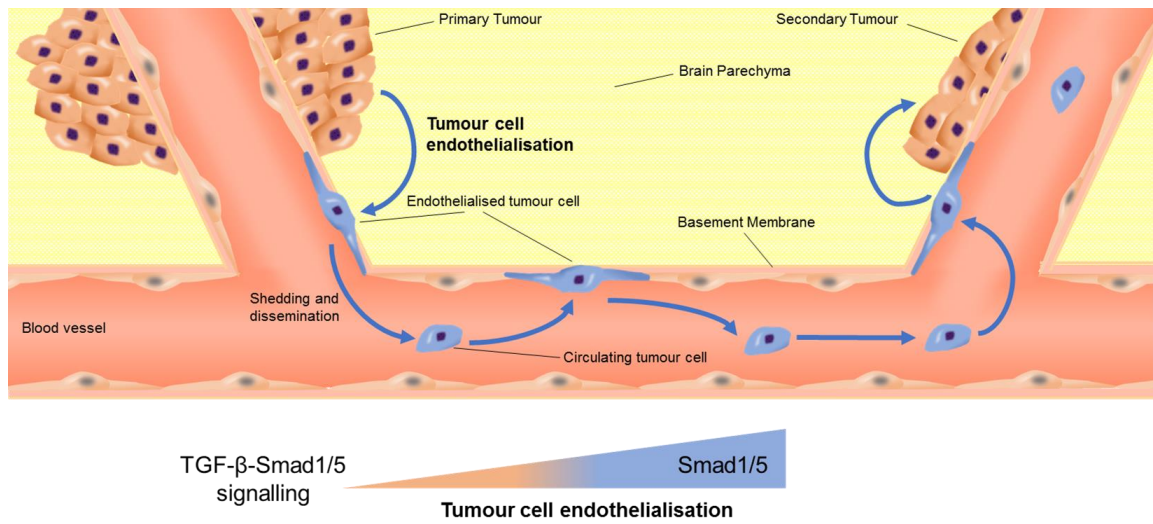


Table 5.1 Patients presenting with gaps in the tumour vasculature

	ESM1	MECOM	ENPEP	EPAS1	GFAP
Patients with blood vessel gaps (%)	86.4 n=22	87.5 n=24	73.3 n=15	60.0 n=15	100 n=7

Supplementary Figure Legends

Supplementary Figure 5.1. Adenovirus transduction efficacy in glioblastoma cell lines.

A, Glioblastoma cell lines were transduced with varying MOI of Ad-CMV-Tom and the percentage of Td-Tomato cells was observed using a Leica fluorescence microscope. n=3 per cell line.

B, Representative images of glioblastoma cells transduced with 500 MOI Ad-CMV-Tom.

C, U87MG cells were transduced with Ad-Smad6 and Ad-Smad7 was monitored over 14 days. 2.5×10^4 transduced cells were seeding into a 24 well plate 24hrs prior to immunofluorescence. Cells were fixed in 3.7% paraformaldehyde and permeabilised in 0.1% Triton-X. Cells were probed with primary anti-M2 antibody (1:1000) and secondary ALEXA-546 antibody (1:5000). Images were captured using a Leica fluorescence microscope (200X magnification). Images are representative of 3 independent experiments

D, Quantitation of Ad-CMV-Tom expression percentage and relative intensity on day 2, 7 and 14 post-transduction.

Supplementary Figure 5.2. ALK1 inhibitors and SMAD1/5 signalling.

A, U87MG cells were concomitantly transduced with 500 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC and seeded at a density of 5×10^3 cells in a 96 well plate. Cells were serum starved and treated with 2ng/mL TGF- β along with either IgG control or 10 μ M PF-03446962 for 6hrs. Cells were lysed, and luciferase activity measured.

B-C, U87MG cells were concomitantly transduced with 500 MOI Ad-BRE-FLUC and 100 MOI Ad-CAGA-GLUC and seeded at a density of 5×10^3 cells in a 96 well plate. Cells were

serum starved and treated with 2ng/mL TGF- β or 20ng/mL BMP7 (C) along with increasing concentrations of LDN-212854.

Supplementary Figure 5.3. A second glioblastoma with *FGFR3-TACC3* fusion gene also presents with tumour cell incorporated into the vasculature endothelium.

In situ hybridisation (ISH) and H&E images were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP) (<https://glioblastoma.alleninstitute.org/>). Images are representative of a single patient with confirmed *FGFR3-TACC3* fusion gene.

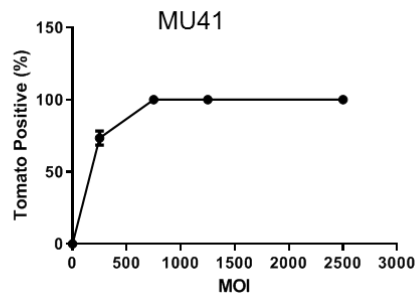
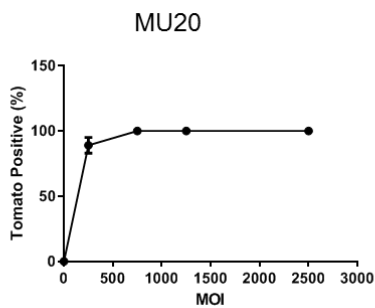
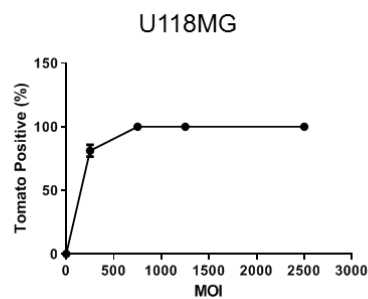
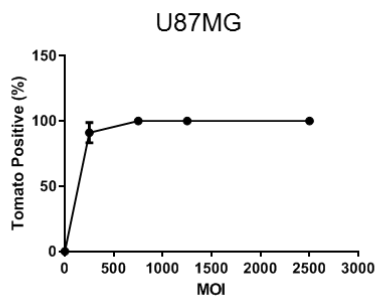
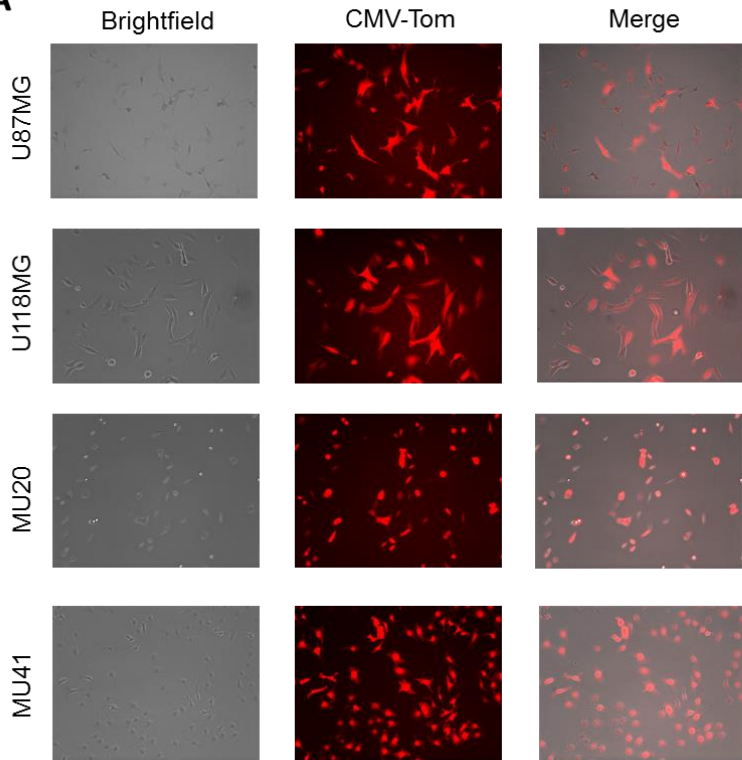
A-B, A tumour blood vessel with *FGFR3-TACC3* or *GFAP* positive ISH staining, arrows indicate positive staining within the endothelium (left panel). Pseudo-colouring of image shown in the left panel for *FGFR3-TACC3* or *GFAP* positive cells (middle panel). Nearest H&E section (right panel).

C-E, A tumour blood vessel showing endothelial marker enriched ISH staining, arrows indicate negative staining within the endothelium (left panel). Pseudo-colouring of image shown in the left panel for endothelial enriched markers positive cells (middle panel). Nearest H&E section (right panel).

Supplementary Figures

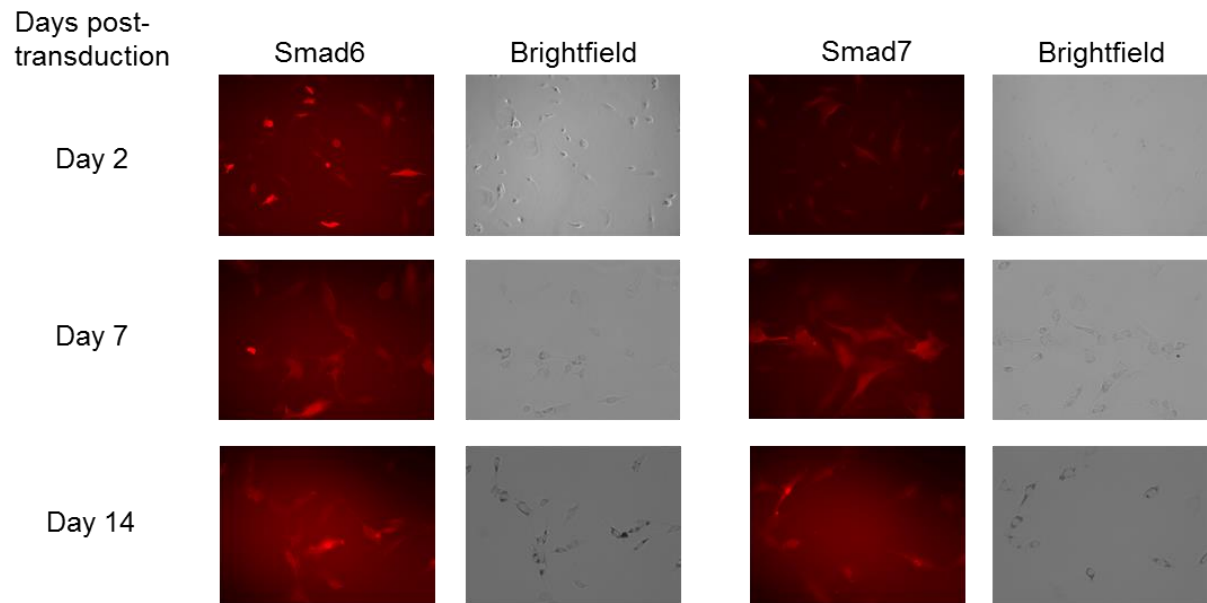
Supplementary Figure 5.1

A

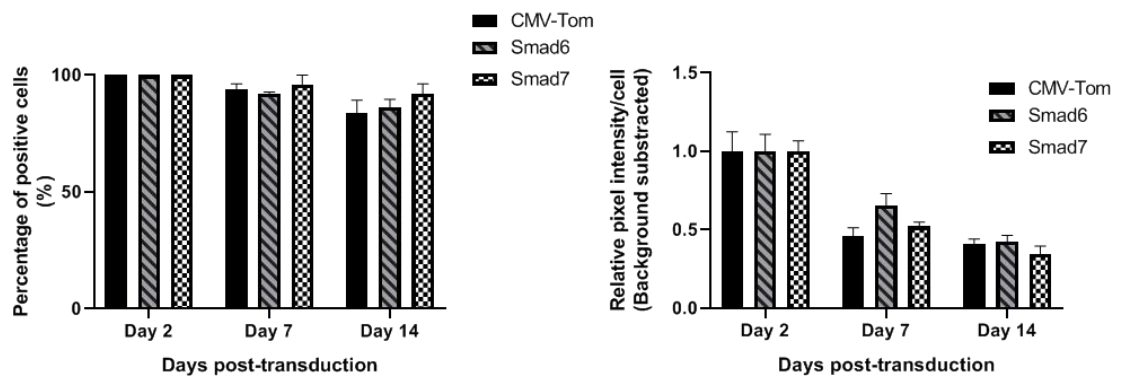


Supplementary Figure 5.1 (continued)

B

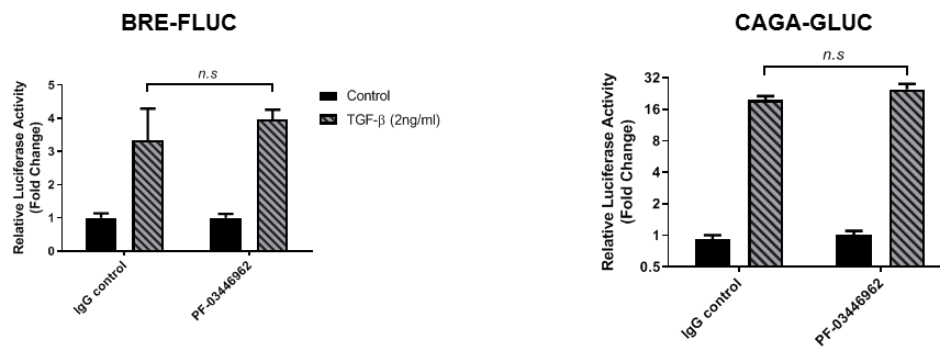


C

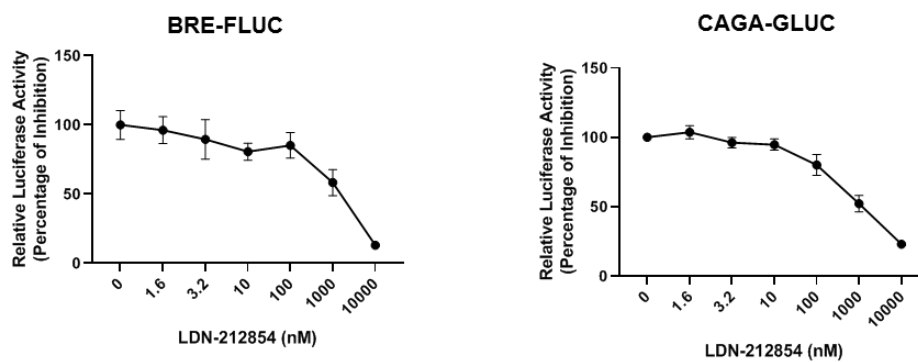


Supplementary Figure 5.2

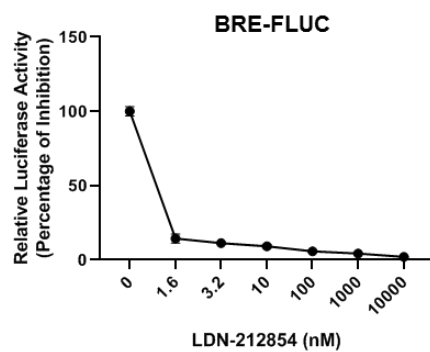
A



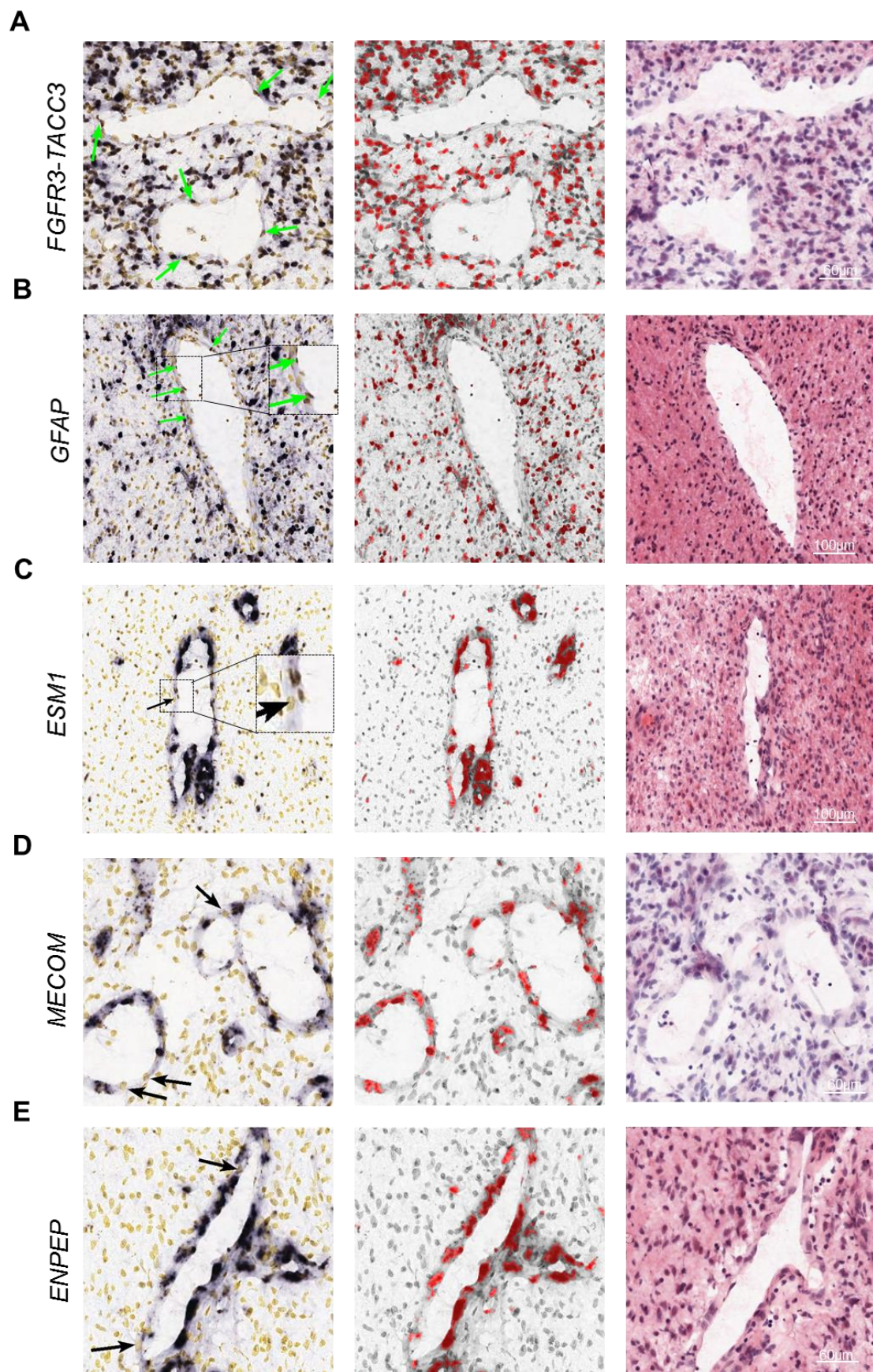
B



C



Supplementary Figure 5.3



General Summary

Despite its aggressive nature and disproportionate morbidity and mortality, glioblastoma has lacked the significant treatment advancements achieved in other aggressive solid cancers. The most advanced form of therapy, the Stupp protocol, has remained the standard of care for 15 years consisting of surgery, radiotherapy and adjuvant chemotherapy, providing a small survival increase, first reported in the EORTC-NCIC phase III clinical trial. Early registry database analyses have also reported an improved survival outcome in the real-world population comparing pre- and post- Stupp time periods, which has supported the standardised use of this protocol. Our study, however, questions these findings, demonstrating that survival outcomes for glioblastoma were already improving prior to the Stupp protocol. Significantly, with the exception of complete surgical resection, all treatment modalities only conferred a short-term survival improvement, up to 18-20 months, following which future survival probability was independent of treatment status. Consistent with this finding, relative survival rates for long-term survivors (>2 years relative survival) remain unchanged since 2000, 5 years before the Stupp protocol, culminating in survival and therapeutic stagnation over this time. The finding that treatment status is not a predictor of survival in the long-term survival cohort, highlights the lack of specificity of treatments for glioblastoma progression. While the short-term survival improvements can be considered to be due to local primary tumour control, eventual tumour progression and recurrence, which is the likely cause of death in long-term survivors, appears to be a clinically distinct setting to that of the primary tumour. The time-dependent implications of treatment in the real-world glioblastoma survival outcomes, suggests there are different biological mechanisms driving primary tumour development and late tumour recurrence. The success of future therapies will be limited based on the understanding of the key molecular driving tumour recurrence.

The importance of understanding the main drivers of tumour recurrence has clearly been observed in the almost 0% success rate in therapeutic development for glioblastoma, even with the most advanced forms of specific targeted therapies. Problematically, these therapies demonstrate promising pharmacological effects in preclinical animal studies, suggesting a discordance between preclinical models and patient glioblastoma biology. Existing models provide limited evidence for efficacy for tumour recurrence, resulting in long and expensive clinical trials based on an incomplete biological understanding. We therefore, developed an animal model to better recapitulate the progression of glioblastoma and recurrence, as a consequence of tumour infiltration. Remarkably, through advanced single tumour cell detection techniques, our model uncovered tumour cells present in all areas of the supratentorial brain, presenting glioblastoma as a systemic disease of the brain. This extensive infiltration presents the probability that these residual cells have the potential to form recurrence glioblastoma and may be the source of the recalcitrant nature of glioblastoma. While this animal model may provide important information for future therapeutic development, its more current significant application is in the use of uncovering tumour recurrence biology.

There is a need to re-examine our understanding of glioblastoma biology and tumour recurrence. The extensive and unique vascularity observed in glioblastoma have been long associated with poor clinical survival and disease progression. Despite these findings, the antiangiogenic inhibitor bevacizumab has failed to treat glioblastoma despite confirmed on-target effects. Our study provides an alternate explanation for the importance of the tumour vasculature for disease progression. Our findings suggest that glioblastoma cells, through the acquisition of an endothelial cell phenotype, integrate into the tumour vasculature whereby they disseminate into circulation and extravasate in distal regions of the brain, presenting as a novel model of glioblastoma infiltration. We have shown that transdifferentiation into

endothelial-like cells is regulated by an expanded TGF- β -ALK1-Smad1/5 signalling pathway in some glioblastoma cells. Importantly inhibition of this signalling pathway reduces overall distal tumour dissemination around the supratentorial brain, establishing a molecular mechanism for this novel mode of tumour infiltration. These findings provide a functional and relevant pathway for haematogenous dissemination in glioblastoma development. Although the TGF- β -ALK1-Smad1/5 presents as a potential novel signalling pathway for therapeutic development, recent research has suggested that glioblastoma is molecularly preordained, having acquired all driver mutations for tumour development and recurrence at the time of diagnosis. Given these findings, glioblastoma may have infiltrated widely throughout the brain at the time of diagnosis, providing little room for intervention to prevent extensive tumour infiltration. This concept goes some way to explain the inevitable tumour recurrence witnessed in Walter Dandy's radical hemispherectomy experiments in an attempt to cure glioblastoma. Early detection and diagnosis may be required to provide the best hope for therapeutic intervention and long-term survival outcomes.

Glioblastoma biology has guided treatment research since its early classification in the late 1920s. During this course remarkable progress has been undertaken expanding on the genetic and molecular profile of glioblastoma and the incipient need of cancer cells to acquire traits, allowing for disease development and progression. Despite, these significant research advancements, therapeutic development for glioblastoma is concerningly limited, highlighting a lack of understanding of the true drivers of disease progression. This study has examined the assumptions and implications of the current standard of care and realised barriers to successful treatment focussing on the infiltrative and distal migratory patterns of glioblastoma development. Through analysis of clinical data, animal models and glioblastoma cell specimens we revealed new glioblastoma biology and molecular mechanisms regulating glioblastoma infiltration.

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