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Anti-angiogenic efficacy of Bevacizumab alone and in combination with a dual PI3K/mTOR inhibitor in an orthotopic model of malignant glioma: A multimodal neuro-imaging approach

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfillment of the degree of Doctor of Philosophy

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Candidate Thesis Declaration

I declare that this thesis, which I submit to the RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree from the RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of knowledge, does not breach copyright law, and had not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed ____________________________

Student Number_______________________

Date________________________
Chapter I  Introduction

1.1. Glioblastoma Multiforme
   1.1.1. Classification
   1.1.2. Incidence
   1.1.3. Clinical Presentation
   1.1.4. Diagnosis
   1.1.5. Genetic/Epigenetic Features
   1.1.6. Treatment
1.2. Surgical Resection
1.3. Radiotherapy
1.4. Chemotherapy
1.5. Prognosis
1.6. Glioblastoma Recurrence
1.7. Neovascularization
1.8. Bevacizumab
1.9. PI3K/mTOR Inhibition
1.10. Combination Strategies
1.11. Neuro-Imaging
1.12. Pre-Clinical Glioma Model
1.13. Hypothesis
Chapter II Materials & Methods

2.1 Clinical Study Methods

2.2 *In vitro* Cell Line Culture

2.2.1. Cell lines

2.2.2. Cell line culture

2.2.3. Sub-culturing of adherent cells

2.3. Routine PCR *Mycoplasma* Testing

2.4. Viable Cell Counting

2.5. *In Vivo* Studies

2.5.1. Intracranial (i.c) GBM xenograft models

2.6. Preparation of Bevacizumab and NVP-BEZ235 for *in vivo* Administration

2.6.1. Preparation of Bevacizumab for *in vivo* administration

2.6.2. Preparation NVP-BEZ235 for *in vivo* administration

2.7. Routes of Drug Administration in Vivo

2.7.1. Intraperitoneal (i.p.) administration of Bevacizumab

2.7.2. Oral gavage (p.o.) administration of NVP-BEZ235

2.8. Toxicity Study

2.9. Efficacy Study

2.10. RadioSythesis

2.10.1 $[^{18}\text{F}]$Fluoride ions

2.10.2 3'-deoxy-3'-$[^{18}\text{F}]$fluoro-L-thymidine ($[^{18}\text{F}]$FLT)

2.10.3 O-(2-$[^{18}\text{F}]$Fluoroethyl)-L-tyrosine (L-$[^{18}\text{F}]$FET)

2.11. *In Vivo* Imaging

2.11.1. Bioluminescence (BLI)
2.11.2. *In vivo* magnetic resonance imaging (MRI)
2.11.3. *In vivo* positron emission tomography (PET)

2.12. MRI Quantification
   2.12.1. MRI tumour volume quantification
   2.12.2. MRI vasculature quantification

2.13. PET Quantification

2.14. Histology
   2.14.1. Brain perfusion
   2.14.2. Haemotoxylin and eosin (H&E) staining
   2.14.3. TUNEL staining
   2.14.4. Immunohistochemistry (IHC)
   2.14.5. Light microscopy
   2.14.6. Image processing
   2.14.7. Proliferation index
   2.14.8. Microvessel density

2.15. Bevacizumab Treatment

2.16. Statistical Methods

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**Chapter III**

**Survival Impact of Resection, Molecular analysis and Bevacizumab treatment in an Irish Glioblastoma cohort**

3.1. Introduction
3.2. Study Aims
3.3. Results
   3.3.1. Overall Use
   3.3.2. Sex and Age Distribution
3.3.3. Tumour Classification and Grade
3.3.4. Tumour Location
3.3.5. Performance Status
3.3.6: Resection Rates
3.3.7. Molecular Analysis
3.3.8: Bevacizumab Therapy (Grade III/IV)
3.3.9: Overall Survival
3.3.10: Univariate Analysis

3.4. Discussion

Chapter IV
Toxicity & Survival: Response to a combined Novel Therapeutic Strategy

4.1. Introduction
4.2. Chapter Aims
4.3. Results
  4.3.1 Toxicity I
  4.3.2 Toxicity II
4.4. Overall Survival
4.5. Animal Weights
4.6. Histology
  4.6.1 H&E
  4.6.2. Ki67
  4.6.3. vWF
  4.6.4. TUNEL
4.7. Discussion

Chapter V
Bioluminescence & Pre-Clinical MRI:
In vivo imaging of anti-angiogenic inhibition in combination with a dual PI3K/mTOR Inhibitor

5.1. Introduction
5.2. Chapter Aims
5.3. Results
  5.3.1. Volumetric Analysis
  5.3.2. Cerebral Blood Volume
  5.3.3. Microvessel Cerebral Blood Volume (µCBV)
  5.3.4. Vessel Size Index
  5.3.5. Mean Density Index
  5.3.6. Apparent Diffusion Coefficient (ADC)
5.4. Discussion

Chapter VI

Pre-Clinical PET: Tumour proliferation and angiogenic response to Bevacizumab in combination with a PI3K/mTOR Inhibitor

6.1. Introduction
6.2. Chapter Aims
6.3. Results
  6.3.1. Pre-Treatment $^{18}$F- FET
  6.3.2. Pre-Treatment $^{18}$F- FLT
  6.3.3. Post-Treatment $^{18}$F- FET
  6.3.4. Post-Treatment $^{18}$F- FLT
6.4. Discussion

Chapter VII

Discussion and Future Direction

7.1 Glioblastoma
  7.1.1. Surgery
  7.1.2. Histopathology
  7.1.3. Radiation
  7.1.4. Chemotherapy
  7.1.5. Targeted Therapy
  7.1.6. Response to Treatment
7.2. Hypothesis
7.3. Discussion

7.4. Future Directions

7.4.1. Combination therapies as potential novel agents.

7.4.2. Advanced neuro-imaging techniques

7.4.3. Expansion of the adult Brain Tumour Biobank

7.4.4. Clinically relevant glioma animal models

7.5. Conclusion

Chapter VIII References

Appendix I Ethical Approval

Appendix II Relevant Publications

Appendix III Oral Presentations

Appendix IV Poster Presentations

Appendix V Prizes/Grants Awarded
Abbreviations

ADC: Apparent Diffusion Coefficient.
ASCO: American Society of Clinical Oncology.
ASL: Arterial Spin Labeling.
BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea.
CCNU: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.
CNS: Central Nervous System.
CT: Computer Tomography.
DCE MRI: Dynamic Contrast Enhanced MRI.
DMBT1: Deleted in Malignant Brain Tumours 1.
DSC MRI: Dynamic Susceptibility Contrast MRI.
DWI MRI: Diffusion-Weighted MRI.
EGFR: Epidermal Growth Factor Receptor.
EOR: Extent of Resection.
EPC’s: Endothelial Progenitor Cells.
FA: Fractional Anisotropy.
FDA: US Food and Drug Administration.
FDG: \(^{18}\text{F}\)2′-fluoro-2′-deoxyglucose.
FET: \(^{18}\text{F}\)-Fluoro-Ethyl-L-Tyrosine.
FISH: Fluorescence in Situ Hybridization.
FLAIR: Fluid-Attenuated Inversion Recovery.
FLT: \(^{18}\text{F}\)-3′- Fluoro-3′ Deoxythymidine.
fMRI: Functional MRI.
FOV: Field of View.
GBM: Glioblastoma Multiforme.
GEM: Genetically Engineered Mice.
GTR: Gross Total Resection.
HGF: Hepatocyte Growth Factor.
HIF1: Hypoxia Inducible Factor 1
HIQA: Health Information Quality Authority.
ICD: International Classification of Diseases for Oncology.
IDH1: Isocitrate Dehydrogenase 1 gene.
IDH2: Isocitrate Dehydrogenase 2 gene.
ICP: Intracranial Pressure
iMRI: Intraoperative MRI.
KPI: Key Performance Indicators.
MGMT: O6-methylguanine- methyl-transferase gene.
MMPs: Matrix Metalloproteinases.
MRI: Magnetic Resonance Imaging.
MRS: Magnetic Resonance Spectroscopy.
MTD: Maximal Tolerated Dose.
mTOR: Mammalian Target of Rapamycin.
NOS: Not otherwise Specified.
NSCLC: Non Small Cell Lung Cancer.
PDGFR: Platelet-Derived Growth Factor Receptor.
PDX: Patient Derived Xenografts.
PCR: Polymerase Chain Reaction.
PET: Positron Emitting Tomography.
PFA: Paraformaldehyde.
PGF: Placental Growth Factor.
PIP2: Phosphatidylinositol (4,5)-bisphosphate.
PIP3: Phosphatidylinositol (3,4,5)- trisphosphate.
PI3K: Phosphatidylinositol 3-kinase.
RANO: Response Assessment in Neuro-Oncology.
ROI: Region of Interest.
RPLS: Reversible Posterior Leukoencephalopathy Syndrome.
RTK: Receptor Tyrosine Kinase.
STR: Sub Total Resection.
TGF: Transforming Growth Factor.
TMZ: Temozolomide.
5-ALA: 5-Aminolevulinic
Figure Legend

Chapter I: Introduction

Figure 1.1: Timing and Frequency of Genetic Alternations in the Evolution of Glioblastoma.
Figure 1.2: Regulation of AKT/PKB activation by PTEN.
Figure 1.3: Chemical Structure of NVP-BEZ235

Chapter III: Survival Impact of Resection, Molecular analysis and Bevacizumab Treatment in an Irish Glioblastoma cohort

Figure 3.1: Overall use of Bevacizumab per Annum.
Figure 3.2: Sex Demographics of Bevacizumab.
Figure 3.3: Age distribution of Bevacizumab
Figure 3.4: Tumour Classification.
Figure 3.5: Astrocytoma Tumour Grade.
Figure 3.6: Age distribution in Astrocytomas.
Figure 3.7: Tumour Side.
Figure 3.8: IDH-1 mutation in Astrocytomas.
Figure 3.9: MGMT methylation in Astrocytomas.
Figure 3.10: Bevacizumab Duration of Therapy.
Figure 3.11: Time Interval from First Surgery to start of Bevacizuamb.
Figure 3.12: Time Interval from Surgery to Mortality.
Figure 3.13: Commencement of Bevacizumab to Mortality.
Figure 3.14: Kaplan - Meier Survival Estimates of Overall Survival according to Age.
Figure 3.15: Kaplan - Meier Survival Estimates of Overall Survival according to IDH Mutational Status.
Figure 3.16: Kaplan - Meier Survival Estimates of Overall Survival according to MGMT Status.
Figure 3.17: Kaplan - Meier Survival Estimates of Overall Survival according to Resection Margin.

Figure 3.18: Kaplan - Meier Survival Estimates of Overall Survival according to Location.

Figure 3.20: Kaplan - Meier Survival Estimates of Overall Survival according to Age.

Figure 3.21: Kaplan - Meier Survival Estimates of Overall Survival according to MGMT Status.

Figure 3.22: Kaplan - Meier Survival Estimates of Overall Survival according to Resection Margin.

Figure 3.23: Kaplan - Meier Survival Estimates of Overall Survival according to Age.

Figure 3.24: Kaplan - Meier Survival Estimates of Overall Survival according to IDH Mutational Status.

Figure 3.25: Kaplan - Meier Survival Estimates of Overall Survival according to MGMT Status.

Figure 3.26: Kaplan - Meier Survival Estimates of Overall Survival according to Location.

Figure 3.27: Kaplan - Meier Survival Estimates of Overall Survival according to Age.

Figure 3.28: Kaplan - Meier Survival Estimates of Overall Survival according to IDH Mutational Status.

Figure 3.29: Kaplan - Meier Survival Estimates of Overall Survival according to MGMT Status.

Figure 3.30: Kaplan - Meier Survival Estimates of Overall Survival according to Location.

Chapter IV: Toxicity & Survival: Response to a combined Novel Therapeutic Strategy

Figure 4.1: Animal Weights: Toxicity I.

Figure 4.2: Mean Animal Weights: Toxicity I.

Figure 4.3: Animal Weights: Toxicity II.
Figure 4.4: Mean Animal Weights: Toxicity II.
Figure 4.5: Overall Survival.
Figure 4.6: Animal Weights.
Figure 4.7: Animal Weights- Individual Groups.
Figure 4.8: H&E Staining- representative animal per group (4x magnification).
Figure 4.9: Ki67 Staining- representative animal per group (20x magnification).
Figure 4.10: Proliferation Index via Ki67 Analysis.
Figure 4.11: vWF Staining- representative animal per group (10x magnification).
Figure 4.12: vWF Positive Particles per Field of View.
Figure 4.13: vWF Staining (vessel size) - representative animal per group (10x magnification).
Figure 4.14: Average Area per Vessel (%FOV).
Figure 4.15: TUNEL Staining.
Figure 4.16: TUNEL Staining-representative animal from control group.

Chapter V: Bioluminescence & Pre-Clinical MRI: In vivo imaging of anti-angiogenic inhibition in combination with a dual PI3K/mTOR Inhibitor

Figure 5.1a: Growth of Intracerebral U87MG-Luc2 assessed using Bioluminescence.
Figure 5.1b: Logarithmic BLI Signal for each Individual Mouse/Group.
Figure 5.1c: Logarithmic Mean BLI Signal ± SEM.
Figure 5.2a: Pre-Treatment Growth Assessment of Intracerebral U87MG-Luc2 using T2- weighted MRI.
Figure 5.2b: Post-Treatment Growth Assessment of Intracerebral U87MG-Luc2 using T2- weighted MRI.

Figure 5.3: Post-Treatment Median Tumour Volume ± range.
Figure 5.4: Post-Treatment Cerebral Blood Volume obtained using R2*MAP sequence.

Figure 5.5: Post Treatment Ipsilateral Median Cerebral Blood Volume ± range.

Figure 5.6: Post-Treatment Microvessel Blood Volume obtained using R2 sequence.

Figure 5.7: Post -Treatment Ipsilateral Median Micro-Vasculature ± range.

Figure 5.8: Post-Treatment Vessel Size Index obtained using VSOP.

Figure 5.9: Median Vessel Size Index ± range.

Figure 5.10: Mean Density Index obtained using VSOP post contrast MRI.

Figure 5.11: Median Density Index ± range.

Figure 5.12: Mean Apparent Diffusion Coefficient ± SEM.

Figure 5.13: Correlation Coefficient MDI v vWF (Pearson’s correlation analysis).

Chapter VI: Pre-Clinical PET: Tumour proliferation and angiogenic response to Bevacizumab in combination with a PI3K/mTOR Inhibitor

Figure 6.1: Control group - Pre & Post treatment PET imaging.

Figure 6.2: BEZ235 - Pre & Post treatment PET imaging.

Figure 6.3: Bevacizumab - Pre & Post treatment PET imaging.

Figure 6.4: Combined Tx - Pre & Post treatment PET imaging.

Figure 6.5: Pre - Treatment Mean $^{18}$F- FET ± SEM.

Figure 6.6: Pre - Treatment Mean $^{18}$F- FLT ± SEM.

Figure 6.7: Post - Treatment Mean $^{18}$F- FET ± SEM.

Figure 6.8: Post - Treatment Mean $^{18}$F- FLT ± SEM.

Figure 6.9: Correlation Coefficient $^{18}$F- FLT v Ki 67 (Pearson’s correlation analysis).

Figure 6.10: Correlation Coefficient $^{18}$F- FET v MDI (Pearson’s correlation analysis).
Figure 6.11: Correlation Coefficient $^{18}$F- FET v VSI (Pearson’s correlation analysis).
Table Legend

Chapter II: Material and Methods

Table 2.1: Ki-67 Immunohistochemistry Preparation.

Chapter III: Survival Impact of Resection, Molecular analysis and Bevacizumab Treatment in an Irish Glioblastoma cohort

Table 3.1: Age Demographics of Bevacizumab Administration.
Table 3.2: Tumour Classification.
Table 3.3: Tumour Grade.
Table 3.4: Tumour Class in Each Age Group.
Table 3.5: Age Distribution in Astrocytomas.
Table 3.6: Tumour Side.
Table 3.7: Tumour Site.
Table 3.8: Pre-operative Performance Status (Grade 3/4 Astrocytomas only).
Table 3.8: Resection/5-ALA Correlation (Grade III/IV Astrocytoma).
Table 3.9: IDH-1 Mutation in Astrocytomas.
Table 3.10: IDH-1 Mutation in Glioblastoma (Age Distribution).
Table 3.11: MGMT Methylation in Astrocytomas.
Table 3.12: MGMT Methylation in Astrocytomas (Age Distribution).
Table 3.13: Bevacizumab Number of Cycles.
Table 3.14: Bevacizumab Duration of Therapy.
Table 3.15: Time Interval from First Surgery to Start of Bevacizumab Therapy.
Table 3.16: Time Interval from Surgery to Mortality.
Table 3.17: Overall Tumour Grade/Mortality Correlation.
Table 3.18: Tumour Grade/Mortality Correlation (Bevacizumab to Death)
Table 3.19: Glioblastoma Data.
Table 3.20: Glioblastoma Univariate Analysis (Bevacizumab to Death).
Table 3.21: Glioblastoma Univariate Analysis (First Surgery to Death).
Table 3.22: Secondary Glioblastoma Demographics.
Table 3.23: Secondary Glioblastoma Univariate Analysis (Bevacizumab to Death)

Table 3.24: Secondary Glioblastoma Univariate Analysis (First Surgery to Death).
Summary

The treatment of glioblastoma represents one of the main oncological challenges of the 21st century. Despite intensive therapeutic efforts, the median survival remains 15-18 months.

Initially, we conducted a single center retrospective study, at the National Neurosurgical Center in Beaumont Hospital, examining the use of bevacizumab in an homogenous Irish glioblastoma population. We demonstrated the importance of tumour location, MGMT (O6-methylguanine- methyl-transferase gene) methylation, IDH (Isocitrate Dehydrogenase) and extent of resection (EOR) as prognostic factors in the setting of recurrent glioblastoma. Our findings also supported the hypothesis that bevacizumab should not be withheld in elderly patients of good performance status. However, no phase III trials have demonstrated an overall survival benefit primary or recurrent glioblastoma setting, treated with bevacizumab. Current evidence suggests that glioblastoma cells are able to circumvent anti-angiogenic therapy, such as bevacizumab (Bev) and develop resistance to targeted monotherapy via activation of complex molecular escape pathways such as PI3K/mTOR pathway, thereby leading to a paradoxical increase in tumour cell invasion. As a result, it is important to assess bevacizumab based combination treatment strategies in order to improve therapeutic outcomes and enhance our molecular understanding of tumour dynamics.

Implementing advanced clinical neuro-imaging techniques (MRI, PET) we mechanistically interrogated the anatomical, physiological, biochemical and vascular properties of glioblastoma in response to a novel drug treatment strategy, in a reproducible orthotopic model. Our working hypothesis was that the combination of a novel dual PI3K/mTOR inhibitor (BEZ235) and bevacizumab would convey potent anti-tumour effects in comparison to monotherapy strategies.
Treatment with bevacizumab resulted in a pronounced decrease in tumour volume (T2-weighted MRI). No further reduction on tumour volume was observed with the BEV/BEZ235 combination compared with BEV monotherapy. The proliferation index markers (Ki67 & [18F]FLT) supported the observations. Using ΔR2* and ΔR2 values, the bevacizuamb/BEZ235 combination significantly reduced tumour blood volume and tumour microvessel volume in comparison to bevacizuamb alone. Microvessel density index was further reduced in animals treated with the combination, supported by von Willebrand factor (vWF) immunohistochemistry. [18F]FET uptake was reduced following treatment with bevacizumab alone, but the addition of BEZ235 in the combination group did not further reduced [18F]FET uptake. vWF immunohistochemistry demonstrated that the mean tumour vessel size was increased in all groups.

**Conclusion:**

Advanced translational neuroimaging techniques provided information on mechanism of action of the drug combination and its potential clinical responses. We have demonstrated that treatment with a BEV/BEZ235 combination could reduce peritumoral oedema reducing the requirement for corticosteroids. Translational studies, similar to this, may help to predict more accurately the clinical potential of the bevacizuamb/BEZ235 combination regimen as a novel therapeutic approach in neuro-oncology.
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As a holder of a BSc (Physiology) and a medical graduate of RCSI, I was always very keen to carry out a higher degree in the subspecialty of Neuro-Oncological Surgery. I was extremely fortunate that Professor Hill and the Beaumont Cancer Research Trust, kindly agreed to provide funding for the thesis. My genuine thanks goes to all of the committee members of this board.

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Annette’s expertise in the field of tumour imaging and angiogenesis was paramount in my successful application for a funded research fellowship at the EIMI from 4th of October- 23rd December 2012. I consider this opportunity one of the highlights of my career. EIMI is a world class pre-clinical imaging centre led by Professor Andreas Jacobs, who not only was gifted in this field but also
secure my lodgings for the 3 months with Dr. Volker Hesselmann as well as providing me with a “set of wheels” in the bicycle capital of the world! The world famous christmas markets meant I was never alone for too long, with a constant flow of family and friends, sampling the “Heidelbeer Gluhwein”. This wonderful experience allowed me develop strong friendships with fellow researchers. In particular, I would like to thank Dr. Thomas Viel, a post-doc from Paris, for his help with individual aspects of the project.

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Overall, this was invaluable experience that I will never forget and will only enhance my career as a Neurosurgeon.

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Dedication

This thesis is dedicated to my wife Gráinne, my newborn son Kealan, and parents Brendan & Bernice for their unwavering support, patience and encouragement.
Chapter I

Introduction
1.1 Glioblastoma Multiforme

1.1.1 Classification

Glioblastoma is defined under the WHO 2016 classification into:

1) Glioblastoma, IDH-wildtype (9440/3) incorporating giant cell glioblastoma (9441/3), gliosarcoma (9442/3) and epitheloid glioblastoma (9442/3).
2) Glioblastoma IDH-mutant (9445/3).
3) Glioblastoma, NOS, a diagnosis that is reserved for tumours where IDH evaluation cannot be performed (9440/3).

1.1.2. Incidence

Cancer of the brain and CNS account for approximately 1.2% of all cancers diagnosed in the Republic of Ireland annually. Glioblastoma has a reported incidence rate of 3.17 per 100,000 and is the most common primary brain tumour in the adult population accounting for 51% of all primary brain and CNS gliomas, with a male predisposition. It is predominantly a disease of adults and older population. Disease incidence increases with age, with a mean age of presentation of 53 - 64 years. Conversely, paediatric glioblastoma is rare, accounting for 3% of all CNS tumours in children. The incidence of glioblastoma is higher in white caucasians in comparison to other ethnicities with a 40-50% reduction in the incidence rate in smaller ethnic population subtypes, reflecting the socio-economic differences in under-reporting in some regions. The majority of glioblastoma tumours (>70%) are located in the frontal and parietal lobes with a predilection for the right hemisphere.
1.1.3. Clinical Presentation

Gliomas present through a wide variety of signs and symptoms and can be correlated to the site of origin. The clinical presentation can either be of a slow insidious nature or present in an acute neurosurgical emergency with raised intra-cranial pressure. Headache is the major presenting symptom in 30-40% of patients\textsuperscript{12,13}. The features of a brain tumour headache are generally nonspecific and vary widely with tumour location, size and rate of growth\textsuperscript{14-16}.

The headache is usually bilateral, but can be on the side of the tumour\textsuperscript{17,18}. The headache associated with brain tumours have similar characteristics to tension-like headaches with a reported incidence of 40-77\%\textsuperscript{18,19}. Nausea and vomiting accompany the headache in 40-60\% of cases\textsuperscript{19-22}. Although headache is a common symptom of a brain tumour, it is infrequently seen in isolation with 66\% of patients complaining of more than one symptom\textsuperscript{13}. Other common salient features include monoparies, gait disturbance, aphasia, memory deficits, visual problems, hemiplegia\textsuperscript{12,13}. Seizures are common in diffuse gliomas, occurring in 50–90\% of low-grade astrocytoma patients and in 20–50\% of glioblastoma patients\textsuperscript{23}. Although seizures are more frequent in low-grade gliomas, the epileptogenic manifestations are more difficult to control in high-grade gliomas\textsuperscript{24}.

Conversely those with more subtle signs have a longer period without detection compromising surgical and radio-therapeutic intervention. In a retrospective study, involving 133 glioblastoma patients, the survival of patients with disabling acute presentation appeared better during the initial 18 months of follow-up, however this benefit was not replicated at later time points\textsuperscript{12}. In the Republic of Ireland, KPI’s directed by HIQA ensure patients diagnosed with a malignant
brain tumour are transferred to a tertiary neurosurgical centre, for definite treatment, within 7 calendar days.

1.1.4. Diagnosis

Following the initial clinical assessment, patients with suspected intra-cranial lesions are further investigated by various neuro-imaging modalities. Significant advancements have taken place in the field of neuro-radiology since the sentinel CT scan was preformed in 1971\textsuperscript{25}.

Although CT is sufficient in showing the majority of intra-cranial lesions\textsuperscript{26}, MRI is the radiological investigation of choice. MRI has set standards in the imaging of brain tumours by allowing better topographic diagnosis and delineation of tumour extension. T1 gadolinium-enhanced imaging is used to assess the leakage of contrast agent through the blood–brain barrier, commonly disrupted in high-grade glioma, and T2-weighted imaging is used to estimate oedema development\textsuperscript{27}. Typically, following intravenous administration of contrast, a nodular or irregular ring enhancing lesion with a central hypo-density (representative of necrosis) with surrounding oedema is highly suggestive of a glioblastoma\textsuperscript{28}. The definitive diagnosis, however, of a glioblastoma (sub-typing and grading according to WHO recommendations) relies on the morphological assessment of stained tissue sections. Increased cellularity, nuclear pleomorphism and atypia, high mitotic count as well as microvascular hyperplasia or necrosis (often pseudopalisading) are histological characteristics of glioblastoma\textsuperscript{1}. Historically, necrosis within a malignant glioma was often viewed as the sole criterion for the diagnosis of glioblastoma. Research has emphasized that vascular proliferation and necrosis are biologically linked, so that either feature can be used for the diagnosis of glioblastoma\textsuperscript{29,30}.

This approach often allows for a confident diagnosis, but the process should nevertheless be an informed one, established in a multidisciplinary setting with
knowledge of clinical information, neurosurgical guidance, radiologic findings and histological features. It is envisaged that future tumour classification schemes will formally incorporate clinical, imaging and molecular genetic criteria.

1.1.5. Genetic/Epigenetic Features

Modern neuropathology serves a key function in the multidisciplinary management of brain tumour patients. Owing to the recent advancements in molecular neuro-oncology, the neuropathological assessment of brain tumors is no longer restricted to provide information on a tumour’s histological type and malignancy grade, but may be complemented by a growing number of molecular tests for clinically relevant tissue-based biomarkers. Genetic and epigenetic aberrations that have gained significance in the molecular diagnostics of gliomas include LOH 10q, deletions of chromosome arms 1p and 19q (oligodendroglioma), promoter hypermethylation of the MGMT gene, TP53 mutations and the mutation status of the IDH1 and IDH2 genes.

The majority of glioblastoma develop after a short clinical history and without evidence of a less malignant precursor (primary or de novo GBM), under the recent 2016 WHO classification nomenclature designated as glioblastoma wildtype. Conversely, progression from a low-grade diffuse or anaplastic astrocytoma (secondary GBM) is classified as mutated glioblastoma. Despite differences in clinical history and genetic, epigenetic and expression profiles, primary and secondary glioblastomas are histologically largely indistinguishable, except that extensive necrosis is more frequent in primary glioblastomas and oligodendroglioma components are more frequent in secondary glioblastomas.
The most frequent genetic alterations in primary glioblastomas are LOH 10q, followed by EGFR amplification, \textit{p16}^{\textit{INK4a}} homozygous deletion, \textit{TP53} mutations, and \textit{PTEN} mutations (Figure 1.1).

**Figure 1.1: Timing and Frequency of Genetic Alternations in the Evolution of Glioblastoma. Modified from Ohgaki et al\textsuperscript{33}**
MGMT

The MGMT gene located at 10q26 is frequently silenced by promoter hypermethylation in diffuse gliomas which has been pinpointed as an epigenetic mechanism reducing MGMT expression levels and is the genetic hallmark of a primary glioblastoma. The MGMT gene encodes a DNA-repair protein that removes alkyl groups from the O\textsuperscript{6} position of guanine, an important site of DNA alkylation. The restoration of the DNA consumes the MGMT protein, which the cell must replenish. Left un-repaired, chemotherapy-induced lesions, especially O\textsuperscript{6}-methylguanine, trigger cytotoxicity and apoptosis\textsuperscript{35,36}. High levels of MGMT activity in cancer cells create a resistant phenotype by blunting the therapeutic effect of alkylating agents and may be an important determinant of treatment failure.

Importantly, an association between MGMT promoter methylation and the response of malignant gliomas to alkylating chemotherapy using nitrosourea compounds\textsuperscript{37}, temozolomide\textsuperscript{38}, or a combination of both has been observed\textsuperscript{39}. Based on MGMT promoter methylation studies in a subpopulation of patients involved in the EORTC/NCIC 22981/26981 trial\textsuperscript{38,40}, Hegi et al. reported that patients treated with radiotherapy and temozolomide, and whose tumors had a methylated MGMT promoter (which is seen in approximately 40\% of primary glioblastomas) survive significantly longer when compared with patients whose tumors lacked MGMT promoter methylation\textsuperscript{38}. In this land-mark paper, MGMT promoter methylation did not significantly influence survival in patients treated with radiotherapy alone, suggesting that the MGMT hypermethylation is predictive for favorable response to chemotherapy.

LOH 10q

While LOH 10q occurs at similar frequencies in primary and secondary glioblastoma, LOH 10p occurs almost exclusively in primary glioblastoma leading to complete loss of chromosome 10\textsuperscript{41}.
Many glioblastomas show loss of one entire copy of chromosome 10, but LOH studies identified at least 3 commonly deleted regions, ie 10p14-p15, 10q23-q24 and 10q25-pter, suggesting the potential of several tumour suppressor genes\textsuperscript{42,43}. Overall, LOH 10q is associated with a poor outcome\textsuperscript{44,45,46}. The \textit{DMBT1} gene is considered to be one of the candidate tumour suppression genes on 10q (10q25.3-26.1)\textsuperscript{47}. It is homozygously deleted in 13%-38% of glioblastomas\textsuperscript{47,48}. The genomic structure of \textit{DMBT1} has recently been elucidated and points to a possible role in evolution of chromosomal instability\textsuperscript{49}.

**PI3K/PTEN/AKT pathway**

Interestingly, a discrepancy exists between LOH for the chromosomal region containing the \textit{PTEN} gene (10q23) and the frequency of \textit{PTEN} mutations (25%). \textit{PTEN} is a phosphatase and regulator of cell cycle progression and apoptosis via the PI3K-AKT pathway and is frequently lost in glioblastoma. EGFR, or other growth factors receptors, become activated upon binding of growth factors (EGF,TGF-\textalpha) and recruits PI3K to the cell membrane. PI3K converts PIP2 to PIP3 (Figure 1.2). \textit{PTEN} inhibits the PIP3 signal thereby inhibiting cell proliferation\textsuperscript{50}. Mellinghof et al demonstrated that a loss of \textit{PTEN} in GBM lead to the development of resistance to therapy with EGFR kinase inhibitors such as gefitinib\textsuperscript{50}. The presence of \textit{PTEN} mutations is not associated with prognosis of glioblastoma patients, particularly in the TMZ era\textsuperscript{51}. 
EGFR

EGFR at 7p12 is the most frequently amplified and overexpressed gene in primary glioblastomas. EGFR is a transmembrane receptor that binds to extracellular ligands such as EGF and TGF and transduces a proliferative signal. EGFR amplification has been identified as a genetic hallmark of glioblastomas. The predictive value of EGFR amplification remains unclear. In previous studies, EGFR amplification was associated with poorer survival of glioblastoma patients. One study of 97 patients showed a lack of predictive value of EGFR amplification; similarly, a meta-analysis of seven previous studies (395 glioblastoma cases) did not detect a significant predictive value of EGFR amplification. Shinojima et al. reported that EGFR amplification was a significant unfavorable predictor for overall survival in glioblastoma patients and that the EGFR gene status was a more significant prognostic factor in patients less than 60 years of age.
Other studies reported that *EGFR* amplification was a predictor of longer survival only in older glioblastoma patients\(^{60,61}\). Simmons *et al.* reported that *EGFR* overexpression was associated with poorer survival of glioblastoma patients younger than the median age and that *EGFR* overexpression was negatively associated with survival in cases without the *TP53* mutation\(^{62}\). Similarly Choi *et al.* demonstrated a significant deleterious effect on survival of glioblastoma patients\(^{63}\). Conversely, a large population-based study indicated that the presence of *EGFR* amplification does not affect survival of glioblastoma patients at any age, however the EGFR amplification was not detected in any glioblastoma patients < 35 years of age\(^{33}\).

*EGFR* rearrangements are also common, the most frequent variant being *EGFRvIII* consisting of an 801-bp in-frame deletion of exons 2–7 that results in a constitutively activated truncated receptor protein lacking the ligand-binding domain\(^{64}\). *EGFRvIII* represents about half of the rearrangements and is identified in 20–30% of unselected primary glioblastomas and 50–60% of the *EGFR* amplified glioblastomas\(^{65}\). This variant of EGFR strongly and persistently activates the PI3K signaling pathway providing critical information for cell survival, proliferation, and motility.

Predictably, increased *EGFRvIII* signaling was associated with a generally poor response to radiation and chemotherapy\(^{66,67}\). However, a benefit resulting from the combined treatment by *EGFR* inhibition with standard therapies (TMZ and radiation therapy) is disputed and as yet the clinical benefit of the use of *EGFR* inhibitors in glioblastomas has been disappointing\(^{68,69}\). Attempts to identify additional biomarkers predictive of response to *EGFR*-related therapies suggested that tumors with *EGFRvIII* and intact *PTEN*\(^{50}\) or with *EGFR* amplification and low levels of phosphorylated AKT\(^{70}\) were more likely to respond to the small molecule tyrosine kinase inhibitors erlotinib or gefitinib.
Studies have demonstrated the anti-EGFRvIII peptide vaccine CDX-110 increased progression-free and overall survival in EGFRvIII-positive glioblastoma patients when added to radio-chemotherapy, although existing patents currently prohibit the use of this antibody for clinical purposes.

**p16INK4a/CDK4/RB1 pathway**

The p16INK4a/CDK4/RB1 pathway is important for the control of progression through G1 into the S phase of the cell cycle. The p16INK4a gene binds to cyclin-dependent kinase 4 and inhibits the cyclin-dependent kinase 4–cyclin D1 complex. This complex phosphorylates the RB1 protein, thereby inducing release of the E2F transcription factor that activates genes involved in the late G1 and S phases. In glioblastoma, disruption of the p16INK4a gene occurs through a homozygous deletion. Inactivation of genes in this pathway is common in both primary and secondary glioblastomas. The CDK4 gene (located at 12q13-14) is amplified in approximately 15% of high grade gliomas, particularly in those without p16INK4a deletion. Survival analysis of CDK4 amplification revealed a highly significant association with a worse clinical outcome. Literature suggests that tumours without p16INK4a deletion or CDK4 amplification, CDK6 amplification occurs suggesting that the two proteins can functionally compensate for each other.

Findings regarding the predictive value of p16INK4a homozygous deletion have been inconsistent. In an analysis of 46 cases, Kamiryo et al. reported that homozygous p16INK4a deletion was a significantly unfavorable criterion for survival of glioblastoma patients. Another study showed that homozygous p16INK4a deletion was associated with shorter survival only in a subgroup of glioblastoma patients < 50 years of age. In a large population-based study (715 GBM cases), no predictive value of homozygous p16INK4a deletion was demonstrated. 

34
TP53/MDM2/p14ARF pathway

The TP53 gene (located at 17p13.1) encodes a transcription factor that plays an important role in several cellular processes, including the cell cycle, response to DNA damage, apoptosis, cell differentiation, and neovascularization\(^8^5\). The MDM2 gene (at 12q14.3-q15) induced by wild-type p53, encodes a 54kDa protein, inhibiting the ability of wild-type p53 to activate its transcription pathway\(^8^6\). In addition, MDM2 promotes the degradation of p53\(^8^6\).

The p14\(^{ARF}\) (part of the CDKN2A locus, at 9p21) gene encodes a protein that directly binds to MDM2 and inhibits MDM2 mediated p53 degradation and transcriptional silencing\(^8^7\). Thus, loss of normal p53 function can result from altered expression of any of the TP53, MDM2 or p14\(^{ARF}\) genes.

Primary glioblastoma is closely associated with the absence of p53 mutation and the presence of gene amplification such as that of EGFR, whereas secondary glioblastoma is associated with the presence of p53 mutation and the absence of gene amplification\(^8^8,8^9\). Data on the predictive value of TP53 mutations in glioblastomas are contradictory. Although some clinical studies showed no association between TP53 status and outcome of glioblastoma patients\(^6^2,9^0\), Schmidt et al.\(^5^7\) analyzed 97 glioblastoma cases and found that the presence of TP53 mutations was a favorable prognostic factor. Conversely, Ohgaki et al.\(^3^3\) demonstrated, the presence of TP53 mutations was predictive of longer survival. However, age-adjusted multivariate analysis revealed no difference in survival between patients with and without TP53 mutations. In addition, FISH and reverse transcription–PCR expression studies have shown coexistence of p53 mutation and EGFR amplification in subsets of primary glioblastoma tumours\(^9^1,9^2\).
Mutations in the gene encoding the human cytosolic NADPH-dependent IDH1, an enzyme that participates in the citric acid cycle, were originally identified in 2008 employing large scale sequencing analysis of 22 glioblastomas. Under physiological conditions, IDH1 and IDH2 are involved in multiple metabolic processes, such as lipid synthesis, cellular defense against oxidative stress, oxidative respiration, and signal transduction. There are different hypotheses on the role of IDH1 and IDH2 mutations in gliomagenesis, including an effect on the stabilization of HIF-1α, up-regulation of other genes involved in angiogenesis, glucose transport, glycolysis and inhibition of developmental apoptosis.

Point mutations affect the highly-conserved arginine residue at codon 132 (R132) in IDH1 gene and, rarely, the homologous arginine residue at codon 172 (R172) in IDH2 gene. IDH1 mutations are detected in >70% of low-grade gliomas and >80% of secondary glioblastoma, especially in younger patients, whereas they are uncommon in primary glioblastoma (<10%). In the landmark paper by Parsons et al, IDH1 mutations were associated with a prolonged overall survival in glioblastoma patients. Subsequent studies analyzing the whole spectrum of diffuse gliomas confirmed the strong association between IDH1 mutation and improved outcome.

In contrast to the strong prognostic significance, several studies reported a lack of predictive significance of IDH1 mutations in gliomas for response to chemotherapy. However, in a retrospective study involving 86 secondary glioblastomas, SongTao et al, found that an IDH mutation was an independent predictor of response to TMZ. Indeed as our understanding of IDH mutations increases, the possibility of IDH1 mutation as a potential biomarker for distinguishing “pseudo” progression from true progression has been hypothesized.
At the genetic level, the presence of IDH1 mutations in diffuse gliomas is strongly correlated with TP53 mutation or 1p/19q deletions as well as MGMT promoter methylation and is inversely correlated with the loss of chromosome 10 and EGFR amplification\textsuperscript{104,105}. This latter finding underscores the hypothesis that primary and secondary glioblastomas are genetically distinct entities, despite their histological similarities.

**1p/19q**

1p/19q genetic status has been examined in malignant gliomas since Cairncross et al. described the clinical implications of 1p/19q co-deletion in patients with anaplastic oligodendroglioma\textsuperscript{106,107}. Prospective randomized clinical trials have validated associations between combined 1p/19q co-deletion and prolonged overall survival of patients treated with radiation therapy with or without chemotherapy\textsuperscript{108,109}. This alteration results from the combined deletion of entire 1p and 19q after unbalanced translocation between chromosomes 1 and 19 [t(1:19)(q10;p10)]\textsuperscript{110,111}. Molecular genetic studies of glioblastoma with an oligodendroglial component have shown heterogeneous genetic alterations, with a variable frequency of losses on chromosomes 1p and 19q, the genetic hallmark of oligodendrogliomas\textsuperscript{80}. While this co-deletion is rarely found in primary glioblastoma, its prognostic value remains undetermined. Current literature would suggest that 1p/19q co-deletion does not correlate with improved survival, with 19q deletions negatively impacting on survival\textsuperscript{112,113}. Interestingly, Holdhoff et al. illustrated that 1p/19q co-deletion was felt to be the most influential test with respect to clinical decision making in patients with glioblastoma, despite its infrequency in primary glioblastoma\textsuperscript{114}.
1.1.6. Treatment

Since 2005, the recommended standard of care involves safe maximum cytoreductive surgery with subsequent radiotherapy plus continuous daily TMZ for 42 days (75mg/m$^2$/day) followed by six cycles of adjuvant TMZ (150–200mg/m$^2$/day for 5 days during each 28-day cycle), otherwise known as the “Stupp Protocol” $^{40}$.

1.2. Surgical Resection

The goals of surgery for malignant glioma are to establish a histological diagnosis and to achieve maximal safe cytoreduction to reduce ICP. The first reported case of glioma resection was performed by Rickman Godlee in 1884 $^{115}$. The oncologic principle of total tumour resection achieved by complete excision with a clear margin has improved survival drastically in many other solid organ malignant tumours. However, this is harder to achieve in glioma surgery due to potential neurological deficits that may be incurred with wide margin resection, especially when the tumour is situated near/in the eloquent cortex. The invasive and widely infiltrative nature of high-grade gliomas makes curative resection impossible. This is supported by the fact that even hemispherectomy has been associated with a poor survival rate. Hemispherectomy as a means to achieve total glioma resection was pioneered by Walter Dandy in 1928 $^{116}$, but despite this procedure, patient survival in the early 1930s was reported to be less than 2 years $^{117-119}$.

Literature from the 1990’s found little evidence to support the hypothesis that aggressive surgical management significantly prolonged survival $^{120-123}$. The perception of resection as beneficial is largely based on a collection of data from retrospective and observational studies that assessed survival or disease progression by extent of resection $^{124-131}$. It is well established that younger patients with a favourable KPS score receive more extensive resections as well as tumours which are superficial or more “accessible” and therefore have a
better outcome. Berger et al demonstrated a survival benefit if GTR is achieved at recurrence, regardless of the initial EOR, suggesting that patients with initial STR may benefit from surgery with a GTR at recurrence. It is now recognised amongst the neurosurgical community that extent of resection directly correlates with overall survival.

Due to the limited lifespan of high-grade glioma patients, it is crucial that surgical debulking does not compound any existing neurological deficit. Otherwise, any potential gain from the surgical resection would be offset by the morbidity. Many techniques have been developed to identify eloquent cortex, especially language, motor and sensory cortex. These adjuncts aid in defining the resection limit, and further debulking beyond this limit will likely increase the risk of surgical morbidity.

fMRI has been developed to assist in identifying language and motor centres. Mueller et al compared the location of the fMRI activation with positive responses to intraoperative cortical stimulation and showed that in patients with more than 2 cm between the margin of the tumour and the activation, no decline in motor function occurred from surgical resection. fMRI of tactile, motor and language tasks is feasible in patients with tumours that are near the eloquent cortex, and shows promise as a means of determining postoperative motor deficit risk following surgical resection of frontal or parietal lobe tumors.

iMRI potentially permits greater safety during aggressive resection of tumours by providing real-time images of residual tumour and the surrounding brain. It also leads to greater surgical accuracy by minimising neuro-navigation errors due to intra-operative brain shift. In a study of 137 patients with WHO Grade III-IV gliomas, Nimsky et al found that 66% of patients with Grade III tumours and 28% of patients with Grade IV tumours underwent extended resection with the guidance of iMRI, thereby increasing the percentage of complete resections by 15% and 12% respectively. Unfortunately, this increase is only
marginal because in many cases, the tumour extends into the eloquent brain areas and could not be excised safely. The integrated application of functional navigation on top of iMRI resulted in a lower postoperative morbidity rate, e.g., a transient new neurological deficit of 10.2% and a permanent neurological deficit of 2.9%. Oh et al.\textsuperscript{136} went on to suggest that this may become the standard of care in due time owing to the fact that patients with less residual tumour may respond more favourably to adjuvant chemotherapy with temozolomide. However, this technology has not been universally adopted because of the high costs of iMRI devices and the learning curve necessary to integrate this into practice without significant increases in operative time. Thus, it may not be clear whether this technology is justified and provides clinically significant benefits from its usage.

An awake craniotomy with local cortical electrical stimulation helps identify the eloquent motor cortex, which cannot be reliably mapped out by anatomical landmarks. Employing identification techniques developed by doing awake craniotomy in 65 patients at the Mayo Clinic, Meyer et al.\textsuperscript{137} found that resecting tumour until the onset of neurological deficits resulted in slightly more than half (52%) of the patients having a greater than 90% reduction in T2 signal postoperatively. At the same time, these techniques allow for good functional recovery. Ninety-four per cent of the 48 patients who developed intraoperative deficits achieved a modified Rankin grade of 2 or less at 3-month follow up.

DTI remains the only noninvasive method capable of segmenting the subcortical course of a white matter tract and has rapidly become an important clinical tool that can delineate functionally important white matter tracts for surgical planning\textsuperscript{138}.

The introduction of 5-ALA is an exciting development as a surgical adjunct. 5-ALA is a natural biochemical precursor of haemoglobin that elicits synthesis and accumulation of fluorescent porphyrins in malignant glioma cells. By using a modified neurosurgical microscope (Pentero) to visualize porphyrin
fluorescence, residual malignant glioma tissue can be identified intraoperatively\textsuperscript{139}. A multicentre study has shown that a significantly larger number of "complete" resections defined as absence of contrast-enhancing tumour on early postoperative MRI can be achieved using 5-ALA-guided resection, compared to conventional white light microsurgery\textsuperscript{140}. In 2006, Stummer et al demonstrated an improved progression-free survival in patients allocated to 5-ALA associated resection compared to white light resection of 41\% and 21.1\%, respectively, with patients in the fluorescence-guided group having a median survival of 15.2 months compared with 13.5 months in the control group\textsuperscript{140}.

1.3. Radiotherapy

Radiotherapy is regarded as the single most important factor for improving overall survival in glioblastoma patients. The benefits of postoperative radiation therapy for glioblastoma was first proven in the Brain Tumor Study Group randomized trial in the 1970's\textsuperscript{141}. Median survival was increased to 37.5 weeks from 17 weeks when postoperative radiotherapy was given compared with best supportive care alone. Subsequent dose response studies have established 60 Gy delivered in 1.8- to 2.0-Gy fractions as the best treatment regimen\textsuperscript{142,143}.

In 2005, after the publication of the results of a European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada phase III randomized trial comparing postoperative radiotherapy and postoperative radiotherapy with concurrent and adjuvant temozolomide, the latter was established as the standard of care because it showed statistically significant survival benefit\textsuperscript{40}.

Radiation therapy has also been shown to improve survival in elderly patients as opposed to best supportive care. A RCT demonstrated that patients >70 years old with a histologically confirmed glioblastoma and KPS >70 randomized to receive radiotherapy (50 Gy in 28 fractions) survived longer than those who
received best supportive care (median 7 vs. 4 months)\textsuperscript{144}. Two RCTs have investigated abbreviated courses of radiotherapy for elderly patients to improve convenience and minimize toxicity of protracted radiotherapy\textsuperscript{145,146}. The RCT by Roa et al.\textsuperscript{145} indicated that 40 Gy in 15 fractions was equivalent to 60 Gy in 30 fractions for radiotherapy alone, justifying the option of using less-intensive radiotherapy schedules for older adults with aggressive glioblastoma. Short-course radiotherapy might be especially appropriate for those with poor performance status where survival after standard radiotherapy is known to be extremely short. Although some literature found no difference in survival of patients who received whole-brain versus partial-brain radiotherapy\textsuperscript{146,147}, the latter must be interpreted with caution as full-dose whole-brain RT may be associated with increased toxicity in the elderly patient population.

Ewelt et al demonstrated that in patients >65 years, with a good performance status, benefited from maximum treatment procedures with cytoreductive microsurgery, radiation therapy, and chemotherapy, thus supporting aggressive management in elderly patients of good performance status\textsuperscript{148}.

\subsection*{1.4. Chemotherapy}

Since chemotherapy first became available in the 1950s, numerous agents have been tested in glioblastoma patients. The nitrosoureas, a class of small, lipophilic alkylating agents that include BCNU and CCNU, were the first drugs to demonstrate activity against glioblastoma\textsuperscript{149,150}. These agents add an alkyl group to the guanine residues in DNA, causing crosslinking and preventing replication. Alkylating agents quickly became the backbone of medical glioma therapy, but broad use was limited by relatively severe side effects such as myelosuppression and, in the case of BCNU, pulmonary fibrosis.
When compared with radiation, neither added a significant survival advantage\textsuperscript{151,152}. Therefore, many patients received first-line radiotherapy. Chemotherapy was considered an alternate option, frequently reserved for disease progression. The landmark EORTC 26981 trial, which compared surgery followed by radiotherapy plus concomitant TMZ to surgery followed by radiotherapy alone in glioblastoma showed there was a statistically significant increase in median survival of approximately 10 weeks (12.1 versus 14.6 months, respectively)\textsuperscript{40}, with an apparent increase in 2-year survival in patients receiving concomitant chemo-radiation when compared to those receiving radiation alone (26.5\% versus 10.4\% respectively). Survival is further enhanced in cases of MGMT promoter methylation following therapy with TMZ (21.7 months versus 15.3 months in the EORTC 26981 trial)\textsuperscript{153,154}.

Alternate delivery methods and amplifying responses through modulating MGMT is generating a substantial amount of research worldwide. One strategy to improve the efficacy of TMZ is to increase the effective dose of TMZ the tumor receives via alternate delivery mechanisms such as local delivery via wafer or convection-enhanced delivery (nano-particles). 1,3-bis (2-Chloroethyl)-1-nitrosourea (BCNU) wafer implantation directly into the resection cavity followed by radiation therapy has shown to survival increase to 13.1 versus 10.9 months for placebo patients (p = 0.03), the results of which lead to FDA approval of Gliadel in the treatment of newly diagnosed glioblastoma in March 2003.

Biodegradable BCNU wafers (Gliadel®) remain the only approved interstitial chemotherapy for treatment of newly diagnosed malignant glioma and recurrent glioblastoma today. Surgically, local intracerebral chemotherapy using BCNU wafers is straightforward, and it is not associated with systemic toxicities\textsuperscript{155,156}. However, apart from the expense of Gliadel wafers, several complications have been associated with implantation of the BCNU wafer, including cerebral edema, healing abnormalities, CSF leaks, intracranial infections, seizures, hydrocephalus, and cyst formation. The rates for these adverse events were
well established in two randomized phase III trials that tested BCNU wafers against placebo wafers\textsuperscript{155,156}. The efficacy of the BCNU wafer treatment followed by radiation chemotherapy within multimodal treatment strategies today remains unproven, however some literature would support the hypothesis that there is an additional benefit of using Gliadel with the ‘Stupp type’ regimen compared to the patients treated with the ‘Stupp type’ regimen only\textsuperscript{157,158}.

In order to further enhance surgical resection and overall survival in glioblastoma patients, a phase II trial is currently examining the safety, tolerability and feasibility of combining fluorescence-guided surgical tumour resection with intra-operative chemotherapy in GBM patients eligible to proceed onto chemo-radiotherapy (CRUK/10/009)\textsuperscript{159}.

1.5. Prognosis

The most significant prognostic factors in glioblastoma patients is age, EOR, KPS, molecular status and mental status\textsuperscript{160}. In 2005, the publication of European and Canadian randomized trials (EORTC 26981/22981-NCIC), changed the paradigm for the treatment of patients with glioblastoma\textsuperscript{40}. This study clearly demonstrated that radiotherapy accompanied by temozolomide followed by 6 monthly cycles of temozolomide gives a significant survival benefit with minimal additional toxicity in patients with glioblastoma. The reported median survival was 14.6 months with radiotherapy plus temozolomide and 12.1 months with radiotherapy alone, with respective 2-year survival rates of 26.5% and 10.4%.

Since then, surgery followed by radiotherapy plus concomitant and adjuvant temozolomide has been considered the standard treatment for adult patients with glioblastoma.
1.6. Glioblastoma Recurrence

Despite gross total resection and owing to the infiltrative nature of glioblastoma, 80–95% of recurrences are located within 2 cm of the resection margin typically occurring within 8 months - 2 years post initial treatment. The majority of patients do not survive beyond 1 year after diagnosis of recurrent disease (1-year survivorship is approximately 20%–25%)\(^{161-163}\). General consensus on appropriate treatment regimes remains controversial. Surgery, radiotherapy, chemotherapy and VEGF inhibitors are all used in various treatment strategies, without a significant benefit in overall survival.

Approximately, 1 in 4 patients with progressive or recurrent glioblastoma can be considered for repeat surgery. The documented benefits of re-operation have been derived primarily from retrospective studies with a significant morbidity/mortality associated with a second operation. A more favorable prognosis following surgery for recurrence or progression is associated with a younger age (70 years or younger), a smaller tumor volume (<50 cm\(^3\)), and a pre-operative KPS greater than 80%\(^{164,165}\). Repeat surgery is not recommended for patients with involvement of pre-specified eloquent/critical brain regions\(^{165}\). A controversial practice at the time of repeat surgery is the implantation of biodegradable chemotherapy wafers containing carmustine, which may prolong survival but are rarely used today\(^{166}\).

Re-irradiation remains a palliative option for a select group of patients with recurrent glioblastoma. Patients with a KPS greater than 60%, a tumor size of up to 40 mm, and progression more than 6 months from time of surgery appear to be the best candidates\(^{167}\). The most common approach involves the use of fractionated stereotactic radiotherapy with or without intensity modulation and a median total dose of 30 – 36Gy\(^{168}\). In contrast, stereotactic radiosurgery, which has the advantage of sparing normal tissue, is rarely used in glioblastoma because of the poorly defined target volume. However, in a recent
A retrospective study involving 77 glioblastoma patients Skeie et al demonstrated a median survival after re-treatment was 12 months for the 51 patients receiving GKS compared with 6 months for re-operation only and 19 months versus 16 months from the time of primary diagnosis, with a significantly lower complication rate of 9.8% and 25.5%, respectively\textsuperscript{169}.

The best available evidence on the efficacy of TMZ in recurrent glioblastoma is derived from several phase II clinical trials, although single-arm studies in which the 6-PFS rate was 15% for recurrent GBMs, with an OR rate of 6% and clinical benefit rate of 33\%\textsuperscript{170}. A metronomic schedule of TMZ may achieve significantly higher efficacy than the standard schedule in 6-PFS and clinical benefit and have a favoring trend in 12 month overall survival but RCTs are required to establish the best therapeutic approach\textsuperscript{171}.

In 2009, bevacizumab was granted FDA approval for the treatment of recurrent glioblastoma, based on two seminal papers, demonstrating an increase in 6-PFS and response rate\textsuperscript{171,172}. Currently, bevacizumab has not been shown to increase overall survival in glioblastoma patients and is associated with significant controversy as to its true mechanism of action as well as the inability to accurately measure treatment response with standard neurological imaging.

Neuro-imaging (serial MRI) remains the primary monitoring tool for glioblastoma, with assessments typically performed every 2 to 3 months during treatment and somewhat longer intervals during disease progression free periods. However, conventional neuro-radiological techniques do not always allow a differentiation between radio-necrosis and recurrence\textsuperscript{173,174}, the former appearing with worsening cerebral oedema mimicking the appearance of a recurrence\textsuperscript{175}. These difficulties in discriminating between tumour progression and the effect of treatment can profoundly compromise subsequent patient management.
Typically “pseudo-progression occurs within 2 months post treatment or radiation necrosis if it occurs 3–12 months after radiotherapy\textsuperscript{176}.

Advanced imaging modalities such as MRS, DWI/ADC, and PET have shown promise alone and in combination for correctly distinguishing tumor recurrence from treatment effects. Use of these advanced techniques will become even more critical with the increasing use of new antivascular therapies that decrease the permeability of the tumour vasculature, which reduces accumulation of contrast in the tumor and thus renders conventional contrast-enhanced MRI of even less value.

Furthermore, detailed analysis of the complex molecular pathways in recurrent glioblastoma is also necessary for establishing how future therapeutic strategies should be optimized.

\textbf{1.7. Angiogenesis/Neovascularization}

Glioblastoma tumours are highly vascularized and therefore represent an attractive target for anti-angiogenic therapies. Since Judah Folkmann’s landmark paper in 1971, many cancer subtypes rely on blood vessels for survival and growth. The proven dependence of tumour growth and metastasis on new vessel formation in animal models has provided a powerful rationale for anti-angiogenic strategies for cancer treatment\textsuperscript{177,178}. Glioblastoma vessels are tortuous, disorganized, highly permeable and characterized by abnormalities in their endothelial wall, pericyte coverage and basement membrane\textsuperscript{179–181}.

These tumours have an innate ability to acquire new blood vessels via \textit{co-option, angiogenesis, vasculogenesis, vascular mimicry and intussusception}\textsuperscript{182}.

\textbf{Vessel Co-option:} is the first mechanism by which gliomas achieve their vasculature. This process involves organization of tumor cells into cuffs around
normal microvessels. Holash et al\textsuperscript{183} were the first to definitively demonstrate vessel co-option, using a rat C6 glioma model. Co-opted vessels have been shown to express angiopoietin-2 (ANG-2)\textsuperscript{183,184}. Possible molecular links between hypoxia and vascular co-option include the up-regulation of ANG-2 by hypoxia through HIF-1–dependent mechanisms and the presence of a HIF-1 binding hypoxia response element location identified in the first intron of the ANG-2 gene (ANGPT2)\textsuperscript{185}. In addition, it has been shown that conditioned medium collected from neoplastic cells exposed to hypoxia promotes vascular co-option\textsuperscript{186}. Montana et al recently described a potential role for bradykinin in chemotaxis during vascular co-option in primary brain tumours\textsuperscript{187}. In glioma biopsy specimens, they demonstrated increased expression of bradykinin receptors in regions of tumour, with the highest levels in perivascular regions.

**Angiogenesis:** Vascular co-option is followed by the development of new vessels from pre-existing ones, a process known as angiogenesis. This mechanism is integral to both physiological and pathological processes. A key early event is the proteolysis of the basement membrane and extracellular matrix due to the activity of MMPs. In the presence of ANG-2, VEGF promotes migration and proliferation of endothelial cells and stimulates sprouting of new blood vessels. Acquisition of the tip and stalk phenotypes among endothelial cells exposed to proangiogenic stimuli involves the delta-like 4 (DLL-4)/Notch pathway\textsuperscript{188}. Ephrin-B2 has also been shown to regulate VEGF-induced endothelial tip cell guidance during angiogenesis, similar to its role in axonal guidance\textsuperscript{189}. Other key mediators involved in angiogenesis include integrins $\alpha_3$ $\alpha_1$ and $\alpha_v$ $\alpha_3$, as well as by CD44\textsuperscript{190}. The end result of the neoplastic angiogenic process is a characteristically abnormal vascular network, leading to abnormal perfusion, with excessive leakiness, that can contribute to the breakdown of the blood-brain barrier.

Hypoxia has long been known as a major stimulator of angiogenesis in glioblastoma\textsuperscript{191}. VEGF, which is up-regulated by hypoxia, stimulates
vascularization during embryogenesis and in neoplastic tissues. The VEGF family consists of five members: VEGF-A (generally referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, and PIGF. VEGF exerts its effects on the vascular endothelium through binding to several high-affinity receptors, including VEGFR-1 (also known as FLT-1) and VEGFR-2 (also known as FLK-1 and KDR). The expression of VEGF and VEGFR correlates with the grade of diffuse astrocytomas, is crucial for glioma growth, and displays a temporal and spatial correlation with the angiogenesis seen in human gliomas. Hypoxia induces HIF-1α expression in glioblastoma and is the main molecular basis for the activation of VEGF gene transcription, leading to angiogenesis. The expression level of HIF-1α and VEGF in both human and murine gliomas is intense around areas of necrosis in pseudo-palisading tumor cells, suggesting that this pattern of HIF-1α and VEGF expression is modulated by tumour oxygenation.

**Vasculogenesis:** involves differentiation of circulating bone marrow-derived cells known as EPCs. VEGF, which has been shown to play a critical role in angiogenesis, also contributes to EPC migration and proliferation. Injection of isolated EPCs in a U87 glioma xenograft model showed that EPCs make up approximately 18% of total vessels, suggesting a significant role for EPCs in tumor neovascularization. Although the molecular identity and differentiation lineage of EPCs is debated, these cells have been defined by their expression of progenitor (CD34, CD133) and endothelial (CD31, VEGFR-2) markers. Moreover, Smadja et al found an SDF-1α/CXCR4–mediated increase in EPC migration, whereas inhibition of SDF-1α/CXCR4 signaling inhibited EPC migration, EPC differentiation, and tubule formation in Matrigel, highlighting the role of the SDF-1α/CXCR4 axis in both the migration and differentiation of EPCs. Similarly, the ANG-2/TIE-2 pathway is important for vasculogenesis, and ANG-2 has been linked to the recruitment of EPCs.

**Vascular Mimicry:** is the ability of tumour cells to form functional vessel-like networks and was first described in human melanoma models. In a study of
101 human glioma samples, Liu et al found a correlation between vascular mimicry and WHO tumour grade. Tumours that contained evidence of vascular mimicry, defined immunohistochemically as CD34−PAS+, were more likely to be higher grade and more aggressive, and these patients had shorter overall survival times than those without vascular mimicry. Interestingly, tumours exhibiting vascular mimicry had lower microvascular densities than those that did not, indicating that vascular mimicry provides a complementary neovascularization pathway.\textsuperscript{199}

**Intussusception** also known as “splitting angiogenesis”, describes the formation of a new vessel by vascular invagination, intra-luminal pillar formation and splitting. Vascular intussusception has initially been described in physiological vascular development\textsuperscript{200} but more recently has been expanded to experimental tumors. It has been suggested that sprouting angiogenesis may switch to vascular intussusception to allow rapid development of new vessels\textsuperscript{201}. The molecular mechanisms that drive vascular intussusception are currently poorly understood and whether intussusception occurs or plays a role in human glioma is currently unclear.

Antiangiogenic agents that were first evaluated in glioblastoma included the oral inhibitors thalidomide, lenalidomide and carboxyamidotriazole, as well as the copper-chelating drug penicillamine. The results with these first-generation anti-angiogenic therapies were disappointing showing no additional clinical benefit compared to the standard of care (nitrosourea-based chemotherapy), weak inhibition of VEGF-mediated angiogenesis, and a lack of survival benefit\textsuperscript{202,203}. Recent clinical trials of targeted agents against EGFR or PDGFR — which are over-expressed in glioblastomas and some carcinomas — have largely been disappointing\textsuperscript{204}. As a consequence more recent investigations have focused on newer, more potent angiogenic inhibitors and in particular VEGF inhibition.
1.8. Bevacizumab

The addition of bevacizumab to standard chemotherapy was initially shown to produce significant clinical benefit (PFS or OS) in patients with metastatic colorectal cancer, advanced non–small cell lung cancer, and metastatic breast cancer (FDA approval withdrawn, November 2011). Data from several clinical trials including three prospective studies have established anti-angiogenic therapy with the humanized anti–vascular endothelial growth factor (anti-VEGF) monoclonal antibody bevacizumab (Avastin®; Genentech, South San Francisco, CA), with or without cytotoxic chemotherapy, as an active treatment option for patients with recurrent GBM who have failed previous TMZ and radiation therapy, leading to the FDA approval of single-agent bevacizumab in previously treated glioblastoma.

In the first completed, prospectively designed, single institution, phase 2 trial of bevacizumab and Irinotecan for recurrent glioblastoma, 20 of 35 (57%) patients had at least a partial response, and the PFS-6 rate was 46% (95% confidence interval [CI], 32%–66%)\(^\text{205}\). The multicenter, randomized, non-comparative phase 2 BRAIN study evaluating bevacizumab with or without Irinotecan in recurrent TMZ-experienced glioblastoma reported response rates of 38% (31/82) with combination therapy, with a median duration of response of 4.3 months\(^\text{171}\). The combination of bevacizumab and Irinotecan was associated with a 6-PFS rate of 50% and a median OS of 8.7 months (95% CI, 7.8–10.9 months). In retrospective analysis and additional phase 2 studies, investigator determined response rates with bevacizumab-based combination therapy have ranged between 19% and 62%, and 6-PFS rates have ranged between 30% and 46% in patients with recurrent GBM, representing an apparent and significant improvement compared with historical controls\(^\text{205-209}\).

In addition to its activity when combined with Irinotecan, bevacizumab has also been shown to increase response and PFS when administered as a single
agent in patients with recurrent glioblastoma\(^{171,172}\). In the phase 2 BRAIN study of patients with glioblastoma who relapsed after TMZ and radiation treatment, the objective response rate with single-agent bevacizumab was 28% (24/85), with a median duration of response of 5.6 months\(^{171}\). The 6-PFS with single-agent bevacizumab was 42.6% (95% CI, 29.6%–55.5%), and the median OS was 9.2 months (95% CI, 8.2–10.7 months). In the single institution, prospective phase 2 NCI 06-C-0064E study of 48 patients with recurrent glioblastoma treated with bevacizumab, 71% and 35% of patients achieved radiographic response based on Levin and MacDonald criteria, respectively\(^{172}\). The median PFS was 16 weeks (95% CI, 12–26 weeks), the 6-PFS was 29% (95% CI, 18%–48%), and the median OS was 31 weeks (95% CI, 21–54 weeks).

In general, bevacizumab treatment is generally well tolerated in patients with recurrent glioblastoma, and the bevacizumab-related toxicities are comparable to those that have been characterized in other solid cancers. Reported rates of grade 3 or higher adverse events with bevacizumab in patients with recurrent glioblastoma have ranged between 18% and 66%, and it appears that the rate of serious treatment-related adverse events is lower when bevacizumab is used as a single agent\(^{171,172,212}\).

In the randomized, non-comparative phase 2 BRAIN study in patients with recurrent glioblastoma, the rate of grade 3 or higher adverse events was 46% in patients treated with bevacizumab monotherapy and 66% in patients treated with bevacizumab plus Irinotecan. The most common adverse events attributable to bevacizumab treatment in recurrent glioblastoma include low-grade bleeding (ie, epistaxis), hypertension, impaired wound healing, and proteinuria\(^{171,205,210}\), which are similar to bevacizumab associated toxicities in other cancer types\(^{211-213}\). The majority of these toxicities appear to be due to off-target, class-specific actions of angiogenic inhibition, and reflect disruption of VEGF in normal tissue. The rates of serious adverse events such as gastrointestinal perforation, RPLS, cardiac failure, and wound-healing complications in glioblastoma studies are low (each <2% incidence). While the reported rate of grade 2 or higher bleeding events has been as high as 5.3%,
life-threatening intracranial hemorrhages have occurred in only a small percentage (<3%) of patients treated with bevacizumab\textsuperscript{172,205,210}.

Several mechanisms of action have been suggested for the anti-tumour effects of bevacizumab, including direct inhibition of tumour-associated angiogenesis, a direct anti-glioblastoma effect on VEGF receptor-expressing glioblastoma cells, disruption of the glioma stem cell microvascular niche, and improved vascular function or normalization.

There are a number of practical issues related to treatment administration, combination therapy, contraindications and other safety-related issues, response evaluation, and disease course that are relevant to the use of bevacizumab for glioblastoma. With regard to administration, the recommended dose and schedule of single-agent bevacizumab is 10 mg/kg intravenously every 2 weeks in patients with recurrent glioblastoma. While most studies in recurrent glioblastoma have evaluated bevacizumab (in combination with Irinotecan) on a schedule of 10 mg/kg every 2 weeks or a weekly equivalent dose of 15 mg/kg every 3 weeks, it is not clear what the ideal treatment schedule or dosage of bevacizumab should be because no direct comparisons of different treatment schedules or dose-response studies have been conducted\textsuperscript{214,215}.

Furthermore, there is evidence that anti-VEGF receptor therapy initially normalizes the tumour vasculature / blood brain barrier in preclinical models as well as in patients with glioma\textsuperscript{216,217} resulting in decreased interstitial pressure and improved tumor oxygenation\textsuperscript{218,219}. This increased oxygen delivery is possibly transient and pre-clinical studies found persisting hypoxia in experimental gliomas exposed to inhibitors of angiogenesis\textsuperscript{216,218}. Because the radiographic response criteria used to assess new agents in this patient population involves a reduction in contrast enhancement, a fundamental
challenge has emerged, in terms of accurately monitoring a patient’s response to bevacizumab therapy. Promising imaging modalities to evaluate anti-angiogenic treated patients include VSOP-enhanced blood volume imaging, MRS, PET, and SPECT nuclear imaging.

Of greater concern, however, is the emerging data illustrating an altered clinical relapse pattern. In bevacizumab-treated GBM patients, tumour relapses are often characterized by local, as well as distant infiltration of the brain by tumours that demonstrate increased invasiveness\textsuperscript{220-223}. This significant problem has become more prevalent as the use of bevacizumab has increased in adult glioma patients.

Investigations have been conducted to try to understand why anti-angiogenic therapy may alter tumour biology and promote, for example, an invasive phenotype\textsuperscript{223}. Evidence for similar phenomena after anti-angiogenic therapy in other tumour types has also been reported\textsuperscript{224}. Although metastases are a rare occurrence in glioma biology, treatment with anti-angiogenic therapy has been shown to increase metastatic potential in both breast and melanoma tumours in preclinical models\textsuperscript{225}. Experimental evidence suggests that the escape from anti-angiogenic therapy such as bevacizumab is at least in part linked to four phenotypic/molecular shifts, many of which are controlled by hypoxia. For example, Piao et al, recently demonstrated the proneural to mesenchymal transition in tumours resistant to anti-angiogenic therapy\textsuperscript{226}. Other relevant shifts include heightened invasion, increased vascular co-option, augmented vasculogenesis, and up-regulation of angiogenic factors other than VEGF. Several experiments have shown that anti-angiogenic therapy can increase invasion and, in many cases, vascular co-option.

Rubenstein et al showed in an athymic orthotopic rat model of glioblastoma that anti-VEGF therapy improved outcomes, but resulted in increased perivascular tumour infiltration and vascular co-option evident on histological analysis\textsuperscript{227}. Enhanced cell infiltration after anti-angiogenic treatment has also been reported.
in other tumour models\textsuperscript{228,229}. In 2011, Keunen et al demonstrated anti-VEGF treatment strongly increased tumour cell invasion, which may result from increased hypoxia in the tumour microenvironment\textsuperscript{230}. Anaerobic metabolism was also reflected by an elevation of metabolites associated with glycolysis (lactic acid & alanine metabolites) and the induction of HIF1α protein. In addition, up-regulation of the PI3K- AKT and the Wnt- signaling pathways in the bevacizumab treated group was found to be significant. It is also interesting to note a significant reduction of mitochondria per cell in bevacizumab treated tumours. These are phenomena are often correlated with increased invasion and metastasis in solid tumours\textsuperscript{231}. Therefore the combination of a dual PI3K/mTOR inhibitor and bevacizumab is an attractive targeted therapy approach.

More recently, Aghi et al suggested that c-Met up-regulation supports tumour survival in hypoxia and invasion, features associated with anti-angiogenic therapy resistance; and growth and therapeutic resistance of xenografts resistant to anti-angiogenic therapy\textsuperscript{232}.

The MET pathway is dysregulated in many human cancers and promotes tumour growth, invasion and dissemination. Abnormalities in MET signaling have been correlated with poor clinical outcomes and drug resistance in patients with cancer\textsuperscript{233}, thereby representing another therapeutic strategy. A phase II clinical trial (NCT01113398) is currently recruiting patients combining AMG102 (a human monoclonal antibody that selectively targets HGF, the only ligand for the c-Met receptor) and bevacizumab in recurrent glioblastoma\textsuperscript{234}.

1.9. PI3K/mTOR pathway

One frequently dysregulated pathway is the RTK /PI3K/Akt/mTOR molecular cascade, which is activated by various mechanisms in glioblastoma\textsuperscript{235}. Analysis of 209 glioblastoma clinical samples by the Cancer Genome Atlas group demonstrated that 86% had a genetic alteration (activating mutation or gene
amplification) in the RTK/PI3K pathway. This signaling pathway plays a central role in the regulation of cell proliferation, growth, differentiation, and survival.

Dysregulation of this pathway is frequently observed in glioblastoma, with activation of the PI3K pathway associated with reduced survival of glioma patients as well as increased resistance to radiotherapy. PI3K/mTOR pathway activation occurs due to genetic alterations in several proteins in the PI3K signaling pathway, including p85, p110, PTEN, and AKT.

Inhibition of the pathway distally using rapamycin resulted in paradoxical activation of AKT through loss of negative feedback in a subset of patients, which in turn was associated with shorter time-to-progression during post surgical maintenance rapamycin therapy. Development of next-generation, class Ia–specific PI3K inhibitors with oral bioavailability has led to a resurgence in efforts to therapeutically modulate this pathway. Identification of one particularly effective PI3K inhibitor also led to the discovery of its ability to concomitantly inhibit mTOR. Since then, additional studies have focused on dual inhibition of both PI3K and mTOR in glioblastoma.

One such agent is NVP-BEZ235 (Novartis) which has shown much promise in pre-clinical studies. BEZ235 is a potent dual PI3K/mTORC1/2 inhibitor that inhibits PI3K and mTOR kinase activity by binding to the ATP binding cleft of these enzymes with IC50 values of 4, 75, 7, 5, and 21 nmol/L against p110a, p110b, p110g, p110d, and mTOR, respectively.
Figure 1.3: Chemical Structure of NVP-BEZ235.

It has been extensively studied in preclinical models, including refractory breast cancer, renal cell carcinoma, sarcoma, and NSCLC. In glioma cell lines, treatment with BEZ235 led to G1 cell-cycle arrest, induced autophagy, and reduced VEGF expression. In vivo, BEZ235 significantly prolonged the survival of tumour-bearing mice\textsuperscript{245}. Furthermore, tumour volume was significantly reduced in a xenograft explant model treated with BEZ235\textsuperscript{244}.

1.10. Combination Strategies

Combination strategies incorporating anti-metabolic agents, such as BEZ235 and angiogenic inhibitors remains poorly defined. It has been shown that combination regimens can be associated with excessive acute or delayed toxicities, which often necessitate a reduction of drug doses or of treatment duration\textsuperscript{246}. In a phase II study, treating renal cell carcinoma patients with bevacizumab and temsirolimus, it was observed
that when more patients were treated for longer durations, grade 3 to grade 4 proteinuria was observed in 26% of the patients\textsuperscript{247}, a much higher rate than the 7% proteinuria rate observed with bevacizumab alone in patients with renal cell carcinoma\textsuperscript{248}. The combination of bevacizumab and sorafenib was associated with increased hypertension and proteinuria as well as an increase in hand and foot syndrome. As a result, bevacizumab required a 50% dose reduction (5 mg per kg, administered once every 2 weeks) and sorafenib could only be given either with a 50% dose reduction on an intermittent schedule (200 mg twice a day, 5 days on, 2 days off) or with a 75% dose reduction (200 mg daily). Even at these attenuated doses, further dose reduction was required in almost all patients after 3–4 months of therapy\textsuperscript{249,250}.

1.11. Neuro-Imaging

A high degree of spatial resolution is facilitated using tomographic approaches. CT may be initially used in the diagnostic process. However, MRI is the gold standard for brain tumour imaging\textsuperscript{251}. MRI continues to be refined in pre-clinical models and now provides both high-resolution anatomical information and functional measurements of tumour physiology\textsuperscript{252}.

The conventional approach to measuring tumour response is based on the criteria of Macdonald et al. which reflect the two-dimensional extent of contrast-enhancing tumour (the product of the maximal cross-sectional enhancing diameters in the same plane) as seen on CT or MRI images\textsuperscript{253}. However, following bevacizumab treatment, in 20–30% of all patients, high-grade gliomas progress as non-enhancing tumours with a growth pattern similar to that of gliomatosis cerebri\textsuperscript{254}. Therefore, the assessment of tumour progression during anti-angiogenic therapy using bevacizumab may be difficult when using the standard MRI criteria of Macdonald et al.
To achieve a more reliable assessment of both tumour response and progression by MRI, the RANO criteria were introduced, in 2010. The RANO criteria recommend hyperintensity on FLAIR or T2-weighted MR images as a surrogate for non-enhancing tumour with an intact BBB\textsuperscript{255}. It is, however, difficult to differentiate nonenhancing tumour from other disorders that cause FLAIR/T2 hyperintensity (e.g. radiation-induced gliosis, peritumoral oedema, ischaemia and demyelination)\textsuperscript{256}. Furthermore, the RANO criteria do not quantify the degree of FLAIR or T2 change necessary to define progression. Consequently, recent efforts have been directed toward identifying new biomarkers that enhance peri-operative management, radiotherapy planning and accurately predict treatment response.

Advanced MRI techniques have now been validated to monitor tumour vasculature and study biochemistry of drug responses. MR modalities showing promise in early detection of response to treatment and disease progression include MRS, perfusion/permeability MRI, DWI-MRI and fMRI. Perfusion MRI may be divided into three categories: DSC-MRI, DCE-MRI and ASL\textsuperscript{257}. DSC perfusion MRI allows measurement of cerebral blood volume (CBV), peak height (PH) and percentage of signal intensity recovery (PSR) within the brain\textsuperscript{258}. DCE-MRI assesses tumour perfusion and angiogenesis by monitoring the pharmacokinetic uptake and rinsing of an MR contrast agent within the extracellular space of tumour lesions. Changes in transfer constant (\(K_{\text{trans}}\)) or the initial area under the Gadolinium (Gd) concentration time curve in tumour, blood adjusted (IAUCBA) in one or more malignant target lesions following drug treatment have been used as primary measurements of drug activity\textsuperscript{259}. The ASL technique is capable of measuring Cerebral Blood Flow. ASL facilitates cerebral perfusion mapping, without administration of a contrast agent or the use of ionizing radiation\textsuperscript{260}.

An important tool in tumour diagnosis is DW-MRI. This technique allows quantification of two values: ADC and FA - the diffusion of water in one plane. ADC is often correlated with tumour size (an increase of ADC values suggests response to therapy, a decrease of ADC values is associated with no response.
to therapy$^{261}$. Based on the principles of DW-MRI, DTI tractography indicates white matter fiber tracts inside a tumour and in surrounding tissues$^{261}$. 3D-volumetric sequences may be fused with DTI tractography on an intra-operative navigation computer$^{257}$.

VSOP coated with citrate (VSOP-C184) is an emerging experimental contrast agent which provides enhanced characterisation of the tumour microvasculature$^{263}$. Superparamagnetic iron oxide particle (SPIO) contrast agents remain intravascular for a prolonged period of time and drastically increase the transverse water proton MR relaxation rate, with the SPIO T2 relaxivity being up to 20 times that of Gd-DTPA$^{264}$. VSOP are substantially smaller (4-8nm) than conventional magnetic nanoparticles due to an electrostatically stabilized citrate coating$^{265}$, and therefore can also be used to detect BBB breakdown$^{266,267}$. With restoration of the BBB, Leenders et al showed that intracerebral lesions of Mel57, a human melanoma cell line with no notable expression of VEGF-A, was not detectable in Gd-DTPA MR images, yet were visible in T2 images after intravenous injection of USPIOs$^{268}$. Similar results have been obtained with intracerebral U87 xenografts$^{269}$. Further studies indicate that SPIO-induced T2* values are highly sensitive to small changes in regional blood volume. In a phase I clinical trial, VSOP was demonstrated to be safe and well tolerated in 18 healthy volunteers$^{270}$. Thus SPIO imaging is an attractive alternative to Gd-DTPA imaging especially for detection and delineation of the vasculature of diffuse infiltrative brain tumours$^{271}$. 

PET is a promising molecular neuro-imaging technique that provides metabolic tumour information complementing the CT and MR imaging examinations$^{272}$. Several studies have evaluated PET by using various tracers (eg, $^{18}$F-FDG, $^{11}$C-MET, $^{18}$F-FET, or $^{18}$F-FLT) as a test for aiding the differential diagnosis of suspected glioma recurrence. $^{18}$F-FDG is the most widely used tracer; its uptake correlates with the amount of glucose consumption and the local metabolic rate within the glioma lesion$^{273}$. However, because of high cortical background activity, $^{18}$FDG-PET is not suitable for detection of residual tumour...
after therapy but is of prognostic value in the diagnoses of cerebral lymphoma. Due to the low contrast between tumour and healthy brain tissue with $^{18}$F-FDG, however, more specific tracers have been developed.

Amino acid tracers such as $^{11}$C-MET and $^{18}$F-FET offer higher contrast than $^{18}$F-FDG based on the increased intracellular (endothelial) amino acid use and extracellular matrix production of tumour cells. $^{18}$F-FET PET has a high sensitivity for the detection of brain tumours and is able to differentiate between high-grade and low-grade brain tumours, irrespective of glial or non-glial histology. $^{18}$F-FET PET positive glial brain tumours without contrast enhancement on MRI confirm tracer uptake into biologically active tumour tissue independently of BBB breakdown, correlating with tumour cell density as well as microvascular density and neo-angiogenesis, all biological hallmarks of highly malignant glial tumours. A recent small prospective study demonstrated $^{18}$F-FET PET-derived imaging parameters superior to MRI, utilising the RANO criteria, in predicting treatments responses to bevacizumab therapy.

Furthermore, uptake of $^{18}$F-FLT correlates strongly with thymidine kinase-1 activity, a cytosolic enzyme with high concentration in proliferating cells but low in resting cells. Because cell proliferation rates are higher in malignant glioma cells compared with scar tissue, $^{18}$F-FLT can also differentiate tumour recurrence from treatment-induced necrosis. A pilot clinical study demonstrated a correlation between reduced FLT uptake and improved overall survival in glioblastoma patients treated with a combination of irinotecan and bevacizumab. Thus, $^{18}$FLT-PET has been suggested as an imaging biomarker for predicting survival benefit in response to anti-VEGF therapy in patients with recurrent glioblastoma.

While currently confined to pre-clinical and clinical studies, the multitude of tracers currently under investigation suggests that PET may play an important role in the future for early evaluation of the efficacy of VEGF inhibitors and other targeted agents. Moreover, with the emergence of anti-angiogenic therapies it is
imperative that the most appropriate imaging modalities are used to determine tumour response to treatment. Importantly, in light of the resistance to therapy evident in the clinic and given the most recent data which has emerged to suggest a paradoxical up-regulation of invasive pathways in response to anti-angiogenic treatment, a future therapeutic strategy may include targeted therapies to compromise both angiogenic and invasive pathways. Thus, the ability to sensitively monitor glioblastoma tumour cell invasion in vivo is likely to become increasingly important.

1.12. Pre-Clinical Glioma Models

Animal modeling for primary brain tumours has undergone constant development over the last 70 years, and significant improvements have been made recently with the establishment of highly invasive glioblastoma models. In vivo models provide essential tumour-host interactions, attempting to replicate the heterogeneity of glioblastoma tissue with regard to proliferation, invasion, vascularization, hypoxia, and genetic alterations, all of which constitute critical determinants for therapy response. Mouse models can be classified as chemically induced models, xenograft tumour models or models of spontaneous tumour formation in GEM mice, and help address issues of utmost importance in drug development: toxicity and in vivo anti-tumour effectiveness.

Many in vivo brain tumor models have been established, starting with patient material transplanted into immunocompetent rodents in the 1940s and 1950’s. Overall, these early studies failed to establish reliable models due to low engraftment rates. However, they revealed that the success of tumor engraftment decreases with lower histological grade and that successful transplantability correlates with poor clinical prognosis (thus, with malignancy). With the introduction of athymic nude mice, the reliability of transplantation of different cancers, including gliomas, significantly
improved\textsuperscript{281,282}. To that end, numerous monolayer cell line models of glioblastoma have been established and used in vast numbers of studies. In particular, U87MG, a long established cell line derived from a human grade IV glioma has been used in over 1,700 publications\textsuperscript{283}. The advantages of cell line–based models include, reproducibility with respect to engraftment rate and reliable growth and disease progression. However, as these monolayer cell lines have been in existence since the 1970’s, concerns regarding its clinical significance are now emerging. They show limited single cell infiltration in the brain; and necrosis and microvascular abnormalities characteristic of human glioblastoma are usually absent\textsuperscript{284,285}. Consequently, biopsy spheroid xenograft models, based entirely on fresh brain tumor biopsies, was established in 1999\textsuperscript{286}.

The transplantation of glioblastoma spheroids provides a reproducible tumour uptake rate close to 100% in immunodeficient rats\textsuperscript{287}. Through further adaptation to the rodent brain by repeated transplantation cycles (giving rise to high-generation xenografts) in some of the tumours, the mitotic index increases, angiogenesis is induced, and necrotic areas and microvascular proliferations appear, closely resembling the heterogeneity of its human counterpart\textsuperscript{288}. Our group recently demonstrated a strong correlation between tumour bioluminescence and tumour volume (MRI), using the patient derived xenografts\textsuperscript{289}.

The most recent advancement in pre-clinical glioma models was the advent of an animal xenograft model based on glioblastoma stem cell enrichment. It has been reported that under appropriate conditions, cultured tumour stem cells derived from primary human glioblastomas exhibit genotype, gene expression profile, and biology of their parental primary tumours\textsuperscript{288}. It is conceivable that subpopulations grouped by markers expression such as CD133, CD15, L1CAM, or integrin α6, represent distinct functional entities that contribute to the phenotypes of human glioblastoma and therefore represent attractive xenograft models. For example, it has been demonstrated that in a mouse model of
medulloblastoma, the Patched mutant mouse, a distinct subpopulation of cells expressing CD15 is able to propagate tumours. While glioblastoma stem cell targeting is very much in its infancy, several markers have show strong potential and future work will characterize the full potential of this exciting model.

Although some authors question the predictive value of animal models for clinical therapy, based on the limited extent of translation into more effective therapeutic clinical treatment strategies, xenograft glioma models have made significant contributions to our fundamental understanding of glioblastoma molecular interactions.
1.13. Hypothesis & Aims

Keunen et al demonstrated anti-VEGF treatment strongly increased tumour cell invasion, which may result from increased hypoxia in the tumour microenvironment. Anaerobic metabolism was also reflected by an elevation of metabolites associated with glycolysis (lactic acid & alanine metabolites) and up-regulation of the PI3K-AKT and the Wnt-signalling pathways in the bevacizumab treated group was found to be significant. Therefore the combination of a dual PI3K/mTOR inhibitor and bevacizumab represents an attractive targeted therapy approach.

**Hypothesis:** Combination of a novel dual PI3K/mTOR inhibitor and bevacizumab conveys potent anti-tumour effects in comparison to monotherapy strategies, using a multi-modality molecular imaging approach.

The aims of this study were three-fold:

1) examine prognostic factors and overall survival in patients diagnosed with glioblastoma recurrence treated with bevacizumab in the National Neurosurgical Centre, Beaumont Hospital, Dublin 9, Ireland.

2) Preform quantitative and qualitative analysis of tumour response to a novel dual PI3K/mTOR inhibitor & anti-angiogenic combination approach in a glioma model.

3) Comparative analysis of advanced neuro-imaging modalities (pre-clinical MRI, PET), in a reproducible orthotopic model.
Chapter II

Material and Methods.
2.1 Clinical Study Methods

Patients who underwent surgical resection, molecular immunohistochemistry and were treated with bevacizumab at the national neurosurgical centre in Beaumont Hospital between January 2013 and December 2015 were included in the analyses. Patient information was obtained from the hospital HIPE database. This information was cross-referenced with data from neuropathology, neuro-radiology and weekly neuro-oncology multi-disciplinary team meetings. Mortality figures were obtained from the hospital BHIS database and RIPv.ie.

Statistical analyses were performed using GraphPad and SPSS (SPSS Statistics Version 20.0). Overall survival was analyzed by the Kaplan–Meier method and the log-rank test for comparison of subgroups. To analyze factors that might influence OS univariate and multivariate Cox regression analyses were performed. P values <0.05 were considered to be statistically significant.

2.2 In Vitro Cell Line Culture

2.2.1 Cell lines

The Bioware luciferase-expressing GBM cell line, U87MG-luc2, was purchased from Caliper Life Science (A PerkinElmer Company, Hopkinton, MA, USA).
2.2.2 Cell line culture

U87MG-luc2 cells were cultured in Eagle’s Minimum Essential Medium (EMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 1 % L-glutamine (2 mM), 1 % penicillin/streptomycin (50 units/ml), all from Sigma-Aldrich (St. Louis, MO, USA).

2.1.3 Sub-culturing of adherent cells

Cells were sub-cultured when the monolayer reached approximately 70-80 % confluency. Waste media was removed and cells were washed with sterile Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO, USA). Cells were then enzymatically detached from the surface of the tissue culture vessel following addition of an appropriate volume of a 0.25 % Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA). The flasks, plates or dishes were returned into the incubator for 2-3 minutes and then struck once to ensure total cell detachment. The enzyme was inactivated by adding a volume of complete growth medium greater than or equal to that of the the volume of a 0.25 % Trypsin-EDTA solution. The cell suspension was then transferred to a sterile centrifuge tube and spun at 1000 rpm for 3 min. The resulting pellet was re-suspended in pre-warmed to 37°C culture medium and seeded at the required density in an appropriate culture vessel. Cells were returned to the incubator and maintained at 37°C in humidified air with 5 % CO₂.

2.2 Routine PCR Mycoplasma Testing

*Mycoplasma* is small (0.2 µm) bacteria with no cell wall, which can grow to high concentrations in mammalian cell cultures. There are no obvious signs of Mycoplasma contamination and these bacteria are undetectable by standard
light microscopy. However, this contamination may induce cellular changes to the infected cells, such as changes in metabolism and growth rates. Cell lines and GBM biopsy-based spheroids were therefore routinely tested for Mycoplasma contamination using the polymerase chain reaction (PCR) Mycoplasma Test Kit I/C for Conventional PCR (PromoKine, Heidelberg, Germany) according to the manufacturer’s protocol. Briefly, samples of 1 ml supernatant, derived from cell cultures that were 90-100 % confluent, were centrifuged for 5 minutes at 500 x g to pellet cellular debris. The supernatant was transferred to a fresh tube and centrifuged at a minimum of 14 000 x g for 15 minutes. The pellet was then re-suspended in 100 µl DNA free water. Following preparation of the polymerase/rehydration buffer mix (22 µl of rehydration buffer and 1 µl of Taq DNA Polymerase were used per reaction), 23 µl of polymerase/rehydration buffer mix was added into test reaction tubes to rehydrate the lyophilised components of tubes. Following the addition of 2 µl of sample to each test reaction tube except positive and negative control tubes (2 µl of DNA-free water was added to positive and negative control tubes) the tube content was mixed by flicking the tube and incubated for 5 minutes in room temperature. The following PCR program was entered: cycle 1: 94ºC for 2 minutes, cycles 2-38: 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 40 seconds, cool down to 4-8ºC and tubes were placed in thermal cycler. The reaction components were thoroughly mixed by vortexing the test reaction tubes before loading to 1.2 % standard agarose gel with a 5 mm-comb wells. 8 µl of each PCR reaction was loaded per lane. Electrophoresis was run for 30 minutes at 100 V. Gel evaluation was made under UV lamp. The mycoplasma-positive control and mycoplasma-positive samples showed a band at 270 bp and the internal control band at 479 bp, whereas the mycoplasma-negative control and mycoplasma-negative samples showed only the internal control band at 479 bp. The internal control DNA band at 479 bp indicated a successfully performed PCR.
2.3 Viable Cell Counting

The harvested cells were counted using a haemocytometer (Hawksley, Sussex, UK). For this method, adherent cell lines were enzymatically detached from the flask using 0.25 % of Trypsin-EDTA, whereas GBM spheroids were dissociated. The cell suspension was then pelleted by centrifugation at 1000 rpm for 3 minutes and re-suspended in 5 or 10 ml of complete growth medium depending on pellet size. 100 µl of the cell suspension was placed in an Eppendorf tube and well mixed with 100 µl of trypan blue (Sigma-Aldrich, St. Louis, MO, USA). 10 µl of the cell suspension containing trypan blue was then transferred onto the haemocytometer and the number of live cells (unstained by trypan blue) were counted in 4 sets of 16 corner squares under a phase contrast microscope. To obtain the number of cell per ml, the total count from 4 sets of 16 corner squares was divided by 4, multiplied by 2 to adjust for the 1:2 dilution in trypan blue and multiplied by $10^4$, which accounts for the volume of the haemocytometer. Using this method, a representative value of the viable cell number per ml in the original cell suspension was obtained.

2.4 In Vivo Studies

All animal experiments were licensed by the Department of Health and Children, Dublin, Ireland. The proposed toxicity experiments as well as specific protocols to establish and use intracranial xenograft models were reviewed by the Royal College of Surgeons (RCSI) Research Ethical Committee (REC). All experiments performed in the study were in accordance with the German Law on the Care and Use of Laboratory Animals and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV). NMRI nude mice were housed at constant temperature (23 °C) and relative humidity (40 %), under a regular light/dark schedule. Food and water were freely available.
2.4.1 Intracranial (i.c) GBM Xenograft Models

Animals were anesthetised by intraperitoneal (i.p.) administration of Ketamine (100 mg/kg) (Narketan 10 %, Vétoquinol, Lure cedex, France)/Xylazine (5 mg/kg) (Xylapan®, Vétoquinol, Lure cedex, France). Alternatively, Ketamine (100 mg/kg)/ Midazolam (5 mg/kg) (Roche, Basel, Switzerland) was used. The head of the recipient mouse was secured in a stereotactic frame (David Kopf, Tujunga, CA, USA). Local analgesia/anesthesia (7 mg/kg of Lidocaine or alternatively 8 mg/kg of Marcaine) was applied 3 minutes before a short longitudinal incision was made. A burr-hole was prepared 0.5 mm posterior to the bregma and 2.5 mm to the right of the sagittal suture using a micro-drill. 1x10^5 of U87MG-luc2 cells were implanted into the cerebral cortex of mice. GBM cell lines were slowly injected, using an appropriate Hamilton syringe including 18G needle for spheroid implantation and 27G needle for cell line implantation (Hamilton, Bonaduz, GR, Switzerland) at 2 mm depth below the brain surface. The syringe was left in place for 5 minutes prior to withdrawal. The skin was closed with an Ethilon 3-0 suture (Ethicon Inc., Novartis Animal Health Inc., Basel, Switzerland). After surgery, the animals were placed in a recovery chamber before being returned to their cages. Close observation of the animals was maintained until recovery from anaesthesia and resumption of normal movement around the cage. Following tumour cell implantation, animals were health checked daily with animal weights. Any changes in general condition/ behavior were noted in score sheets.

2.5. Preparation of Bevacizumab and NVP-BEZ235 for in Vivo Administration

2.5.1. Preparation of Bevacizumab for in vivo administration

For in vivo experiments, where NMRI nu-nu mice bearing intracranial U87MG-luc2 tumours, as well as mice included in the Toxicity study were treated with Bevacizumab (Avastin; Roche). Bevacizumab was prepared fresh the morning of dosing, at a concentration of 1mg/ml in a vehicle solution of 0.9% NaCl
(Baxter, Berkshire, UK), each mouse receiving 10mg/kg bodyweight, alternating days for 12 days. Control mice received the equivalent dose of the vehicle only (0.9%NaCl).

2.5.2 Preparation NVP-BEZ235 for *in vivo* administration

NVP-BEZ235 (Selleck Chemicals, Houston, TX, USA) was formulated daily in 1-Methyl-2-pyrrolidinone (NMP; 494496; Sigma-Aldrich, St. Louis, MO, USA) / polyethylene glycol 300 (PEG 300; 202371; Sigma-Aldrich, St. Louis, MO, USA) in a concentration of 1:9 (v/v). BEZ235 (Free Base) was dissolved in NMP on sonication and the remaining volume of PEG300 was added. For *in vivo* toxicity experiments, mice received 25mg/kg or 45mg/kg, daily via oral gavage for 5 days with 2 days off, for a total of 3 weeks. Mice bearing intracranial U87MG-luc2 tumours received 45mg/kg daily via oral gavage for 5 days with 2 days off, for 16 days. Control mice received the equivalent dose of the vehicle only (NMP/PEG300).

2.6 Routes of Drug Administration in Vivo

2.6.1 Intraperitoneal (i.p.) administration of Bevacizumab

Mice were firmly immobilised by pulling up the loose skin on the flanks. The head and body were tilted downward. The right lower abdominal wall was disinfected with 70% isopropyl alcohol swabs (MidMeds Limited, Essex, UK). Freshly formulated Bevacizumab in 0.9% NaCL or vehicle alone was quickly injected into the peritoneal cavity using a 26 gauge 3/8" (10 mm) brown needle (BD Biosciences, Franklin Lakes, NJ, USA). Immediately following drug administration, the animals were returned to their cages and monitored for any adverse effects.
2.6.2 Oral gavage (p.o.) administration of NVP-BEZ235

Mice were firmly restricted by pulling up the loose skin on the flanks to immobilize the head and straight the mouse body. A 0.61mm x 40 mm flexible oral dosing needle (VetTech Solutions Ltd., Cheshire, UK) was inserted down the esophagus and freshly formulated BEZ235 in NMP/PEG 300 (1:9;v/v) or vehicle alone was slowly injected. The needle was carefully withdrawn and immediately following drug administration the animals were returned to their cages and monitored for any adverse effects.

2.7 Toxicity Study

5-6 week old female NOD/SCID (NOD.CB17-Prkdc<sup>scid</sup>/J) mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA) and maintained at the SPF-grade scantainer (Scanbur Technology, Karlslude, Denmark) within the RCSI Biomedical Research Facility (BRF). In each animal facility the animals were allowed to acclimatise for at least 1 week following delivery, prior to commencement of procedures. Phase I of the study (3 weeks), mice received 25mg/kg of NVP-BEZ235 via oral gavage, daily for 5 days, with a 2 day rest period. Simultaneously, the animals received 10mg/kg of Bevacizumab via intra-peritoneal injection, alternating days for 12 days. Phase II of the study (3 weeks), mice received 45mg/kg of NVP-BEZ235 via oral gavage, daily for 5 days, with a 2 day rest period in addition to 10mg of Bevacizumab via intra-peritoneal injection, altering days for 12 days. These mice were monitored daily, using the mouse monitoring score sheets (Appendix I- examining weight, skin, behaviour, breathing etc). At the end of monitoring period, mice were anesthetised and sacrificed by cervical dislocation.
2.8 Efficacy Study

Experiments to examine the efficacy of the combination drug therapy using a xenograft model were performed in accordance with the regulations of the German Committee for Animal Research and approved by the Local Institution Board at the University of Münster. 5-6 week old females NMRI \textit{nu/nu} mice, used for xenograft models, were purchased from Janvier Laboratories (St.Berthevin, France) and maintained in Individually Ventilated Cages (IVC)-system, within the animal laboratory facility. The animals were allowed to acclimatise for at least 1 week following delivery, prior to commencement of procedures. 3 weeks post implantation, the mice received 45mg/kg of NVP-BEZ235 via oral gavage, daily for 5 days, with a 2 day rest period in addition to 10mg of Bevacizumab via intra-pertioneal injection, alternating days for 12 days for a total of 18 days. Control mice received the equivalent doses of the vehicle. The mice were monitored daily, using the mouse monitoring score sheets (Appendix I- examining weight, skin, behaviour, breathing etc). At the end of monitoring period, mice were anaesthetised, the brain was perfused and removed for histological analysis.

2.9. Radiosynthesis

All chemicals, reagents and solvents for the synthesis of the compounds were analytical grade, purchased from commercial sources and used without further purification, unless otherwise specified. Radiosyntheses were carried out using an automated PET tracer synthesizer (TRACERLab Fx FN Synthesizer; GE Healthcare). Separation and purification of the radiosynthesized compounds were performed within the radiosynthesizer by radio-RP-HPLC using a Knauer K-1800 pump (flow=7 mL/min, eluent: water for injection/EtOH 97/3 (v/v), a Knauer K-2501 UV-detector (λ=254 nm), and a Nucleosil 100-10 C18 column (250 x 8 mm$^2$). The recorded data were processed by the TRACERLab Fx software (GE Healthcare).
2.9.1. $[^{18}F]$Fluoride ions

No-carrier-added aqueous $[^{18}F]$fluoride ions were produced on a RDS 111e cyclotron (CTI-Siemens) by irradiation of a 1.2 ml water target using 10 MeV proton beams on 97 % enriched $[^{18}O]$water by the $^{18}O(p,n)^{18}F$ nuclear reaction.

2.9.2. 3′-deoxy-3′-$[^{18}F]$fluoro-L-thymidine ($[^{18}F]$FLT)

In a computer controlled TRACERlab Fx FN Synthesizer (GE Healthcare) the batch of aqueous $[^{18}F]$fluoride ions from the cyclotron target was passed through an anion exchange resin (Waters Sep-Pak Light Accell Plus QMA cartridge, preconditioned with 5 ml 1 M $K_2CO_3$ and 10 ml water for injection). $[^{18}F]$Fluoride ions were eluted from the resin with a mixture of 32 µl 1 M $K_2CO_3$, 200 µl water for injection, and 800 µl DNA-grade CH$_3$CN containing 12 mg Kryptofix® 2.2.2 (K 222) into the reaction vessel. Subsequently, the aqueous K(K 222)$[^{18}F]F$ solution was carefully evaporated to dryness in vacuo. 20 mg (24.1 µmol) of precursor compound (3-$N$-Boc-5′-O-dimethoxytrityl-3′-O-nosyl-thymidine) in 1 ml DNA-grade CH$_3$CN was added and the mixture was heated at 90°C for 10 min. The mixture was cooled to 40°C, 800 µl 1 N HCl was added and the hydrolysis was performed at 105°C for 10 min. After cooling to 40°C 370 µl 2 N NaOH and 400 µl phosphate-buffer (Na$^+$ 1 mmol/ml; PO$_4^{3-}$ 0.6 mmol/ml) were added. The solvents were removed at 90°C in vacuo and after cooling to 60°C 2 ml eluent (water for injection/EtOH 97/3 (v/v)) were added. The mixture was passed through a Waters Sep-Pak Light Alumina N cartridge (preconditioned with 10 ml ethanol and 10 ml water for injection) and purified by the radio-RP-HPLC system of the radiosynthesizer. The product fraction of $[^{18}F]$FLT (retention time $t_R = 6.0 \pm 0.6$ min) was collected in the product vial that contained 2 ml 10 % NaCl. Finally, the $[^{18}F]$FLT solution was dispensed into a sterile pyrogen-free 20 ml glass vial by passing through a 0.22 µm sterile filter (Millipore Millex FG). The total activity of $[^{18}F]$FLT at the end of the radiosynthesis was 1.2-5.6 GBq (2.9 ± 1.8 GBq).
2.9.3. O-(2-[18F]Fluoroethyl)-L-tyrosine (L-[18F]-FET)

For automatic production we used GE TRACERlab MXFDG for L-[18F]-FET as remote controlled synthesizer. The module operates with a disposable cassette which is highly favourable for tracer synthesis due to less cross contamination.

*18F-Fluoride fixation, desorption and drying*

Separation of n.c.a. 18F-fluoride from 18O-enriched water is accomplished via fixation of 18F-fluoride on an anion exchange cartridge (Sep-Pak QMA Light cartridge). Subsequently, the activity is eluted using K222/K2CO3/ACN/H2O solution and dried by azeotropic distillation.

*Nucleophilic fluorination with 18F*

Fluorination of the precursor O-(2-tosyloxyethyl)-N-trityl-L- tyrosine tert-butyl ester was realised in acetonitrile. The mixture was heated to 120°C for 5 min. Then the solvent was evaporated at 85°C.

*Hydrolysis*

Cleavage of both protecting groups was accomplished by adding hydrochloric acid and heating up to 120°C for 10 min.

*Purification and formulation*

L-[18F]-FET was purified by SPE using Oasis WAX Plus, Oasis HLB Plus and Sep-Pak Light Alumina N cartridges. The formulation of the final product is included in the automatic synthesis and the final solution contains a buffer solution to adjust the pH.
Radiochemical/chemical purity:

The radiochemical purity was determined using the following conditions:
Econosil C18, 10μ, 250 x 4.6mm, eluent: H2O/ACN gradient, flow: 1 mL/min, 254 nm, gamma detector.

Enantiomeric purity: The enantiomeric purity of $^{18}$F-FET was analysed using
Crownpak CR(+), 150x4mm, Diacel Chemical Industries, Ltd., eluent: 90%
20mM perchloric acid, 10% MeOH, flow: 1ml/min, gamma detector.

Gas chromatography: Determination of residual solvents was established by
headspace-gas chromatography (incubation: 90°C, 15 min; run: DB-624
column, 40°C, 15 min, N$_2$-flow: 2mL/min, FID).


pH: 5.5-7.5.

Reagent and hardware kits for the synthesis of L-[$^{18}$F]-FET on GE TRACERlab
MXFDG are available at ABX GmbH, Radeberg Germany.

2.10 In Vivo Imaging

2.10.1 Bioluminescence (BLI)

In vivo BLI was performed using the IVIS Spectrum (Caliper Life Science,
PerkinElmer Company, Hopkinton, MA, USA). Approximately 5 minutes
following an i.p. injection of 150 mg/kg of D-luciferin (Caliper Life Science,
PerkinElmer Company, Hopkinton, MA, USA), animals were anaesthetised with
isoflurane/O$_2$ mixture and BLI began 7 minutes after D-luciferin administration.
Isoflurane/O$_2$ anaesthesia was maintained during imaging by nose cone delivery.
In vivo BLI of luciferase expression were attained using a 1-120 seconds exposure time with medium binning, f-stop of 1 and field of view (FOV) B (22.5 cm, when 2 mice imaged at the same time), C (12.5 cm, when 3 mice imaged at the same time). Parameters were adjusted to obtain the in vivo bioluminescent signal above the noise level (recommended > 600 counts) and blow CCD saturation (< 60 000 counts). A region of interest (ROI) was traced around the tumour area manually and corresponding total flux photons/seconds values were obtained for each image.

2.10.2. In vivo magnetic resonance imaging (MRI)

MRI was performed with a 9.4 T small animal MR scanner with 20 cm bore size with a mouse brain surface coil (Bio-Spec 94/20; Bruker BioSpin MRI GmbH, Germany). The system was operated with the software ParaVision 5.1. (Bruker BioSpin MRI GmbH, Germany). Mice were anaesthetized with isoflurane (5% induction, 1-1.5% maintenance, DeltaSelect; Dreieich, Germany) in compressed air/O₂ (70:30, 1 L/min). Body temperature and respiration rate were continuously monitored during the MRI exam. An i.v. catheter (27G) was introduced into one tail vein. The animal was placed prone in the animal cradle with the head fixed by bite bar, nose cone and ear plugs. We obtained T2w RARE anatomical images in the sagittal, axial, and coronal imaging plane: TR = 2000 ms, TE = 50 ms, Rare factor 8, 8 averages (coronal 12), field of view 1.6 cm, matrix 128 x 128 (coronal 192), slice thickness 0.75 mm, resolution 125 x 125 (coronal 83 µm². Perfusion was assessed with a 1 slice ASL experiment at the biggest tumour diameter using a FAIR(Flow sensitive alternating inversion recovery)-RARE protocol (FOV 1.6 cm, slice thickness 0.75 mm, 1 slice, TR/TEeff. 18000/44.8 ms, 22 inversion recovery times (TIR 26.8-5000 ms), inversion slab thickness 3.75 mm, scan time 9 min.

Vessel parameters, tumour microvessel density and tumour vessel size, were determined by acquiring ΔR2 and ΔR2* maps before and after i.v. administration of very small superparamagnetic iron oxide particles (VSOP C184,
Ferropharm, Teltow, Germany) at a dose of 30 mg Fe/kg (injection volume, including dead volume of the injection line, 150-180 µl). C184 is coated with citrate and has a sphere size of 8 nm and a core size of 4 nm. Protocol parameters were adapted from Ullrich RT et al.\textsuperscript{332}

MSME and MGE MR images were obtained with the same geometry (FOV16 mm\(^2\), matrix 64\(^2\), slice thickness 0.3 mm). MSME was acquired with TR=5000 ms and 10 echos, TE=10.9, 21.8, ..., 109 ms. MGE was acquired with TR=1400 ms and 10 echos, TE=4, 8, ..., 32 ms with a 60° hermite pulse. The post contrast image acquisition was delayed by 3 min. Data for an apparent diffusion coefficient (ADC) map with the same geometry were additionally acquired before contrast agent application, with a diffusion-weighted EPI protocol (TR/TE 7500/18.4 ms) with b=0, 300, 800 s/mm\(^2\). Total scan time was approximately 45 minutes per animal.

2.10.3. \textit{In vivo} positron emission tomography (PET)

Mice were anaesthetized with 2 % isoflurane (DeltaSelect; Dreieich, Germany) in O\(_2\), and one lateral tail vein catheter was positioned using a 27 G needle connected to a 15 cm polyethylene catheter tubing. 12 MBq \([^{18}\text{F}]\text{FLT} or [^{18}\text{F}]\text{FET were injected as a bolus (100 µl of [^{18}\text{F}]\text{FLT}[/^{18}\text{F}]\text{FET solution flushed with 100 µl saline) via the tail vein, and subsequent PET scanning was performed. PET experiments were carried out using a high resolution (spatial resolution of 0.7 mm using iterative EM reconstruction including resolution recovery) small animal scanner (32 module quadHIDAC, Oxford Positron Systems Ltd., Oxford, UK) with uniform spatial resolution (< 1 mm) over a large cylindrical field (165 mm diameter, 280 mm axial length). List-mode data were acquired for 20 min starting 70 min after tracer injection for [^{18}\text{F}]\text{FLT, and for 10 min starting 20 min after tracer injection for [^{18}\text{F}]\text{FET.}}\)
2.11 MRI Quantification

2.11.1 MRI tumour volume quantification

PET data were reconstructed into a static frame using an iterative reconstruction algorithm. PET and MR images were co-registered using contours of the skull and head of the mice using the software VINCI (Vinci 3.99; http://www.nf.mpg.de/vinci3/). A volume-of-interest (VOI) approach was used to determine the maximal radiotracer uptake in the tumour. To calculate tumour-to-background uptake ratios, the tumour tracer uptake was divided by the mean tracer uptake in the mirror regions of the tumours drawn in the contralateral hemisphere. MR images were used to segment tumour volumes with Amira.

2.11.2 MRI vasculature quantification

Cerebral blood flow maps were calculated with the Paravision Macro perfusion. Tumour blood flow and parenchymal blood flow at the contralateral site were determined in manually drawn ROIs in the slice with the biggest tumour diameter. The T1 value of blood at 9.4 T was set to 2400 ms. Matlab routine (R2010b) was used for calculation of ΔR2, ΔR2* and ADC maps. Vessel parameters, VSI and MDI were calculated as proposed by Ullrich et al. The ADC map was calculated from diffusion-weighted images with a mono-exponential fit of the signal intensities of the diffusion-weighted images (b0, 300, 800 s/mm²). ROI analysis was performed of the slice with the biggest tumour diameter. ΔR2* maps were calculated with the second echo (8 ms) by

\[
\Delta R_{2*} = \frac{2}{3} \frac{\ln(GE_{pre})}{GE_{post} - GE_{pre}}
\]

ΔR2 maps were determined with the third echo (32.7 ms) by
\[ \Delta R_2 = 0.694 \delta \omega^{2/3} \xi_0^{2/3} \frac{Q^2}{ADC} \frac{R^{-2/3}}{TE} \ln \left( \frac{SE_{pre}}{SE_{post}} \right) \]

\( \xi_0 = \text{Blood Volume fraction}; \quad \delta \omega = 2\pi \gamma \Delta X B_0 = \text{frequency shift}; \quad \# = \text{number of 180° pulses}; \quad R = \text{Vessel Size Index (VSI)} \)

\( \Delta X = \text{changes in the susceptibility}, \quad B_0 = \text{magnitude of the magnetic field}, \quad \gamma = \text{gyromagnetic ratio}. \)

The Micro vessel density index was calculated from

\[ MDI = \frac{\xi_0}{(2\pi R^2)} = 1.327 \frac{Q^2}{ADC} = 1.327 \frac{\Delta R_2}{(\Delta R_2 \times \xi^{2/3})^3} \]

The vessel size index (VSI) was calculated from

\[ VSI = R = 0.425 \frac{ADC}{\delta \omega}^{1/2} (\Delta R_2 / \Delta R_{\text{adj}})^3/2; \]

\( \delta \omega = 2\pi \Delta X B_0 \) with \( \Delta X = 1 \times 10^{-3} \).

\( (\Delta X = \text{susceptibility change}) \)

Median indices of tumor tissue within the skull were compared between groups.

**2.12. PET Quantification**

PET data were reconstructed into a static frame using an iterative reconstruction algorithm. PET and MR images were co-registered using contours of the skull and head of the mice using the software VINCI (Vinci 3.99). MR images were used to delineate the contour of the tumours and to measure their volumes. A VOI approach was used to determine the maximal radiotracer uptake in the tumour. To calculate tumour-to-background uptake ratios, the tumour tracer uptake was divided by the mean tracer uptake in the mirror regions of the tumours drawn in the contralateral hemisphere.
2.13. Histology

2.13.1. Brain perfusion

After the last imaging session, mice were deeply anesthetized and perfused transcardially with saline for 5 min, followed by 4% PFA for 2 min. Brains were post-fixed in PFA overnight.

2.13.2. Haemotoxylin and eosin (H&E) staining

Routine hematoxylin and eosin (H&E) staining and analysis of subcutaneous GBM tumour sections was performed by a qualified staff at the European Institute of Molecular Imaging (EIMI), University of Münster, Germany. Briefly, formalin-fixed paraffin-embedded (FFPE) tissue sections were placed on a heating plate at 65°C for 10 minutes and the melted paraffin was wiped off. Following de-paraffination, performed in xylene (Sigma-Aldrich, St. Louis, MO, USA) or alternatively in Histo-Clear II (National Diagnostics, Atlanta, GA, USA) (2 x 5 minutes), the tissue sections were re-hydrated in descending gradient alcohols (2 x 3 minutes in 100 % ethanol and 2 x 3 minutes in 96 % ethanol/dH$_2$O) and dH$_2$O (1 x 2 minutes). The slides were then stained in acid hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes followed by rinsing in running tap water for 20 minutes and de-colorisation in 1 % acid alcohol for 1 second. Tissue sections were further rinsed in running tap water for 5 minutes and stained with alcoholic eosin Y (Sigma-Aldrich, St. Louis, MO, USA) for 30 seconds, washed with dH$_2$O and de-hydrated in ascending gradient alcohols (2 x 3 minutes in 96 % ethanol/dH$_2$O and 2 x 3 minutes in 100 % ethanol). Following, clearing with xylene or histo-clear II (2 x 5 minutes), the DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA) was used to mount cover slips on the sections.
2.13.3. TUNEL staining

For Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, sections fixed in acetone were permeabilized in sodium citrate solution (0.1 g in 100 ml). Sample sections were incubated 60 min at 37° with 25 µl TUNEL reaction mixture (In situ TUNEL kit assay; Roche Inc.). Positive and negative control sections were treated according to the manufacturers manual. Sections were finally covered with 6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector, Germany).

2.13.4. Immunohistochemistry (IHC)

FFPE tissue sections were placed on a heating plate at 65°C for 10 minutes and melted paraffin was wiped off. Following de-paraffination, performed in xylene (Sigma-Aldrich, St. Louis, MO, USA) or alternatively in Histo-Clear II (National Diagnostics, Atlanta, GA, USA) (2 x 5 minutes), the tissue sections were re-hydrated in descending gradient alcohols (2 x 3 minutes in 100 % ethanol and 2 x 3 minutes in 96 % ethanol/dH₂O) and dH₂O (1 x 2 minutes). Heat-mediated antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) or 1 mM EDTA (pH 8) in microwave (5 minutes at high power, 10 minutes at low power). The sections were then cooled on bench for 20 min and washed in TBS-T (2 x 5 minutes). All slides were blocked with 200 µl per slide of 5 % serum/TBS-T (serum from the same species as the secondary antibody) for 1 hour at room temperature, followed by an incubation with primary antibody for indicated time (Table 2.1). All primary antibodies used in immunohistochemistry (IHC) were diluted as indicated in 1 % serum/TBS (serum from the same species as the secondary antibody).
Table 2.1: Ki-67 Immunohistochemistry preparation

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Incubation Time/Temperature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ki67</td>
<td>Rabbit</td>
<td>1:100</td>
<td>1 h/ RT</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
</tbody>
</table>

Following incubation, all unbound primary antibody was removed by washing the slides in TBST (3 x 5 minutes). The endogenous peroxidases were blocked by incubating the sections with peroxidase blocking reagent (Dako, Glostrup, Denmark) for 10 minutes following washing in TBST (3 x 5 minutes). The appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was diluted as indicated at the Table 1 in 1 % serum/TBS (serum from the same species as the secondary antibody) and applied to each slide for 1 hour at room temperature.

2.13.5. Light Microscopy

Light microscope was used as a standard tool to count cells (via haemocytometer), to study cell morphology during cell culture and to obtain images of tissue sections following H&E or IHC staining. Objective lenses used were between x4 to x40 magnification with an eyepiece x10 magnification. Histological analysis was performed using a Nikon Eclipse 90i light microscope (Nikon, Germany) and the NIS-Elements software package (Nikon, Germany).

2.13.6. Image processing

All images obtained from live/dead viability assay were analysed using ImageJ imaging software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).
2.13.7. Proliferation index

To assess proliferation indices based on Ki-67 staining of intracranial GBM xenografts, 1 image with high density of brown staining were collected within each brain section using 20 times magnification. Ki67-positive cell number and total number of cells per image were quantified manually using cell counter in ImageJ software. Proliferation index was expressed as a percentage of Ki-67-positive cells.

2.13.8. Microvessel density

To assess microvessel density, based on vWF tumour staining of GBM xenografts, vessel counts were performed. Briefly, 3 images with high density of brown staining were collected withing each tumour section at 10 times magnification. Images were overlaid with a 2500^2 grid using ImageJ software. Microvessel density was determined from the number of positively stained vessels that crossed the overlaid grid-lines. Only positively stained structures with clearly identified lumens or structures containing erythrocytes were counted. Positive single cells were not taken into account. Positively stained structures that evidently belonged to the same vessel were counted as one, whereas each degree of branching more than one bend was counted as one more vessel.

For quantification of the average area per vessel, colour images were deconvolved (H-DAB colour deconvolution) and the resulting monochrome images were thresholded. Thereafter, the positive counts per FOV were quantified using the “Analyse particles” plugin. The total area with positive vWF staining inside the FOV was divided by the number of vWF-positive particles per FOV to calculate the average area per vessel in per cent of FOV.
2.14. Statistical methods

Statistical methods used to analyse the difference between groups were performed using GraphPad (San Diego, CA, USA) and SAS (Cary, NC, USA). Data is represented as median ± range, unless otherwise stated. Two sample non parametric median tests were employed. Determined p values when < 0.05 were considered to be statistically significant. (Scale of images: 100μm).
Chapter III

Survival Impact of Resection, Molecular analysis and Bevacizumab Treatment in an Irish Glioblastoma cohort
3.1 Introduction

Glioblastoma is the most common primary adult brain tumour and is associated with a dismal prognosis, despite unprecedented clinical/translational research in recent times. It is a diffusely infiltrating, aggressive brain tumour that has evaded past/current treatment strategies, and remains one of the most difficult oncological diseases to treat in the 21st century. The recommended standard treatment of a combined strategy of maximum cytoreductive surgery, radiotherapy with concurrent and adjuvant temozolomide (TMZ) for adult patients with glioblastoma, the typical survival is only 12-15 months from the time of diagnosis\textsuperscript{40,292,293}, with a median time to recurrence of 7 months\textsuperscript{5}. This necessitates the requirement for significant advances in our understanding and treatment of the disease.

Several patient and tumour variables have been shown to affect the prognosis of patients with glioblastoma including age, pre-operative functional status, peri-operative imaging of the tumour, gene expression analysis and surgical resection.

There is a growing body of evidence supporting the hypothesis that EOR conveys a survival benefit in patients with glioblastoma\textsuperscript{121,122,129,133}. A retrospective analysis combining 416 patients with newly diagnosed and recurrent glioblastoma concluded that a $\geq 98\%$ EOR is necessary to significantly improve survival\textsuperscript{294}. In 2011, Berger et al, demonstrated that a survival benefit could be achieved with an subtotal resection of 78%. In an attempt to maximize EOR, Stummer et al implemented fluorescence guided resection\textsuperscript{140}. The ALA-Glioma Study Group enrolled 260 patients in a prospective, randomized multicenter trial examining intraoperative 5-ALA–mediated tumour fluorescence versus conventional white light for high-grade glioma resection and demonstrated a significant difference in rates of complete resection (65\% for 5-ALA vs 36\% for white light).
Recently, Sawaya et al, targeted the complete resection of contrast enhancing tumour in 876 patients. The median survival time for these patients (15.2 months) was significantly longer than that for patients undergoing less than complete resection (9.8 months; p < 0.001), without any significant increase in postoperative morbidity/mortality\(^{295}\).

In 2016 the WHO re-defined the nomenclature of tumours of the central nervous system, using molecular parameters to reflect modern tumourigenesis and identify prognostic molecular biomarkers\(^{296}\). IDH1/2- wildtype glioblastoma accounts for 90% of cases which correspond to primary/de novo glioblastoma under the 2007 WHO classification. Wildtype - IDH1/2 consist a typical pattern of genetic changes including gain of chromosome 7 (containing PDGFα), loss of chromosome 10 (containing PTEN), EGFR amplification and are associated with poorer survival. Conversely, IDH1/2 mutated glioblastoma (10% of cases) correspond to “secondary” glioblastoma, preferentially occurring in younger patients and reflecting a transformation from a lower grade glioma. Aberrant IDH1 R132H protein (IDH1-mutated) is now an established marker of survival benefit in glioblastoma\(^{93,96,97}\). However, the benefit of IDH1-mutation maybe offset when controlled for tumour location. Kaye, et al, did not show any survival benefit for IDH1-mutated glioblastoma when controlled for location ie (frontal lobe) compared to IDH1- wildtype\(^{297}\).

In addition, MGMT promoter methylation has shown significant survival benefit in glioblastoma patients, first reported 18 years ago\(^{37,298}\). MGMT is a DNA repair protein that removes the alkylation of the O6 position of guanine, the most cytotoxic lesion induced by alkylating agent chemotherapy.

Loss of MGMT expression results in an aberrant methylation of the MGMT promoter region, leading to gene silencing and consequently reduced proficiency to repair DNA damage induced by alkylating agent chemotherapy. Hegi et al. reported that patients treated with radiotherapy and temozolomide, and whose tumours had a methylated MGMT promoter (which is seen in
approximately 40% of wild-type glioblastoma’s) survive significantly longer when compared with patients whose tumors lacked MGMT promoter methylation\textsuperscript{154}.

The survival benefit was reiterated in 2006, in the RTOG 0525/EORTC/North Central Cancer Treatment Group Intergroup Study. Gilbert et al, demonstrated an overall survival benefit in MGMT methylation compared to unmethylated of 23.2 months and 16 months, respectively\textsuperscript{299}.

In the last decade, understanding/interrogating the microenvironment, including tumour proliferation, progression and angiogenesis has grown exponentially. Glioblastomas are highly vascular tumours and therefore represent an attractive target for anti-angiogenic therapies. Angiogenesis leads to the formation of new blood vessels from existing vasculature, characterized by endothelial cell migration and proliferation. The angiogenic “switch” in the tumour environment is mediated by various pro-angiogenic factors such as VEGF, which are released by tumour stromal and endothelial cells. It has also been demonstrated that bevacizumab provides potent inhibition of VEGF, preventing the proliferation and migration of endothelial cells, which in turn downregulates tumor vascularization and results in tumor cell hypoxia and death\textsuperscript{300}. More recent preclinical studies investigating the use of bevacizumab in glioblastoma models have detected normalization of mature blood vessels, microvascular regression, and the inhibition of new blood vessels being formed in tumours\textsuperscript{301}.

As discussed previously, data from several clinical trials including three prospective studies have established anti-angiogenic therapy, with or without cytotoxic chemotherapy, as an active treatment option for patients with recurrent glioblastoma who have failed previous TMZ and radiation therapy, on the basis of an improved PFS only. This lead to the FDA approval of bevacizumab treatment in recurrent glioblastoma in the United States. However, due to the paucity of an overall survival benefit, the EMA rejected the use of bevacizumab for the treatment of
recurrent glioblastoma; although, bevacizumab is administered for off label use as a monotherapy throughout Europe, as a second line therapy. Consequently, this highlights the important debate surrounding the application of appropriate endpoints for clinical trials in this area.

Recently, investigators examined the whether the combination of bevacizumab and lomustine improved OS in patients with first progression of a glioblastoma compared to lomustine alone (EORTC 26101). Wick et al conducted the study based on the overall survival benefit found in the phase II Belob study. The combination therapy showed an increase PFS only with no overall survival benefit. Toxicity was in the expected range with more events in the combination arm.

The current clinical trend appears to reserve bevacizumab for use in recurrent disease, or for patients with moderate or severe neurologic symptoms. Despite clinical ambiguity, bevacizumab remains the only targeted therapy for the treatment of recurrent disease. While for the purpose of clinical trials bevacizumab is commenced at the initial recurrence, ordinarily it is reserved as a last line therapy in neurosurgical centers worldwide. Therefore, we retrospectively examined all patients treated with bevacizumab with intra-cranial tumours at the National Neurosurgical Center in Beaumont Hospital, Dublin.
3.2 Study Aims

The use of bevacizumab in the setting of glioblastoma remains unclear. Several phase III trials have failed to demonstrate any overall survival benefit, both in primary and recurrent glioblastoma. Despite this, the VEGF-inhibitor is still used as a “second line” therapy although no formal license has been approved by the EMA.

The aim of this retrospective study was to perform a quantitative and qualitative analyses, in a heterogenous Irish Glioblastoma cohort, treated at the National Neurosurgical Centre, evaluating:

1) overall incidence & demographics of bevacizumab administration in the National Neurosurgical Centre, Beaumont Hospital, Dublin, Ireland.
2) prognostic molecular immunohistochemistry biomarkers, including MGMT and IDH.
3) tumour response/survival to resection rates and also to 2nd line systemic therapy (bevacizumab).
3.3 Results

3.3.1: Overall Use

105 patients were treated with bevacizumab for intra-cranial tumours between January 2013 and December 2015 (Figure 3.1).

Figure 3.1: Overall use of Bevacizumab per Annum.

Despite the use of bevacizumab in Europe for the treatment of recurrent glioblastoma is off label and represents a minority of therapeutic salvage strategies available in the recurrent setting. Therefore, the use of bevacizumab is increasing in Ireland annually for disease control.
3.3.2: Sex and Age Distribution

Figure 3.2: Sex Demographics of Bevacizumab

Table 3.1: Age Demographics of Bevacizumab Administration

<table>
<thead>
<tr>
<th>Gender</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>72</td>
<td>51.86</td>
<td>55.50</td>
<td>20</td>
<td>84</td>
</tr>
<tr>
<td>Female</td>
<td>33</td>
<td>52.76</td>
<td>54.00</td>
<td>16</td>
<td>74</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>52.14</td>
<td>55.00</td>
<td>16</td>
<td>84</td>
</tr>
</tbody>
</table>
There were 72 (68.6%) males and 33 (31.4%) females. Ages ranged from 16 to 81 with a median age of 55 years, with a peak prevalence among this cohort between age 45 to 70 year olds (Figure 3.3). There were no differences in mean and median ages between male and female.

Figure 3.3: Age distribution of Bevacizumab
3.3.3: Tumour Classification and Grade

Table 3.2: Tumour Classification

<table>
<thead>
<tr>
<th>Tumour Class</th>
<th>Astrocytic</th>
<th>Oligodendroglial</th>
<th>Meningioma</th>
<th>Rad (GBM), Hist (OA)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>66</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>105</td>
</tr>
</tbody>
</table>

The majority of patients treated with bevacizumab were diagnosed with an astrocytoma (96 patients). Patients with an oligodendroglioma and meningioma also received bevacizumab, 5 patients and 1 patient, respectively (Figure 3.4). There was histological/radiological discordance in 3 cases and these patients were treated with bevacizumab (Table 3.2).
Figure 3.4: Tumour Classification

Table 3.3: Tumour Grade

<table>
<thead>
<tr>
<th></th>
<th>WHO Grade 2</th>
<th>WHO Grade 3</th>
<th>WHO Grade 4</th>
<th>Rad Grade 4, Histo Grade 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytic</td>
<td>1</td>
<td>14</td>
<td>81</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>Oligodendroglial</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Meningioma</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rad (GBM), Hist(OA)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2</strong></td>
<td><strong>19</strong></td>
<td><strong>83</strong></td>
<td><strong>1</strong></td>
<td><strong>105</strong></td>
</tr>
</tbody>
</table>
Figure 3.5: Astrocytoma Tumour Grade

The majority of intra-cranial tumours treated with bevacizumab were glioblastoma (77%), of which they accounted for 84% of all astrocytomas (Figure 3.5). A single male patient with a grade II meningioma received bevacizumab. All patients diagnosed with an oligodendroglioma who received bevacizumab were grade III (Table 3.3).
### Table 3.4: Tumour Class in Each Age Group

<table>
<thead>
<tr>
<th>Tumour Class</th>
<th>Total</th>
<th>Astrocytic</th>
<th>Oligodendroglial</th>
<th>Meningioma</th>
<th>Rad (GBM), Hist (OA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 18</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18 to 44</td>
<td>29</td>
<td>26</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>45 to 70</td>
<td>68</td>
<td>62</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Over 70</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>96</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 3.5: Age distribution in Astrocytomas

<table>
<thead>
<tr>
<th>Astrocytic Grade</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Rad: GBM, Path: Grade 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 18</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18 to 44</td>
<td>1</td>
<td>8</td>
<td>17</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>45 to 70</td>
<td>0</td>
<td>6</td>
<td>56</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>Over 70</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>14</td>
<td>81</td>
<td>3</td>
<td>99</td>
</tr>
</tbody>
</table>
64% of patients with an astrocytoma were aged between 45-70, 27% of astrocytoma’s occurred in patients aged between 18-44, with 7 patients over the age of 70. 1 male patient under the age of 18 received bevacizumab (16 years of age) for treatment of a glioblastoma (Table 3.5/Figure 3.6). One patient, aged between 45-70 years old with neurofibromatosis type II who developed a meningioma, also received bevacizumab.
3.3.4: Tumour Location

Table 3.6: Tumour Side

<table>
<thead>
<tr>
<th>Side</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>47</td>
<td>44.8</td>
</tr>
<tr>
<td>Right</td>
<td>56</td>
<td>53.3</td>
</tr>
<tr>
<td>Bi-hemispheric</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Brainstem only</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>100.0</td>
</tr>
</tbody>
</table>

More than half of all tumours (53%) occurred in the right cerebral hemisphere with 45% located in the contralateral hemisphere. One tumour crossed the midline and involved both hemispheres and 1 tumour was infra-tentorial, located in the brainstem (Table 3.6/Figure 3.7)
Table 3.7: Tumour Site

<table>
<thead>
<tr>
<th>Site</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>44</td>
<td>41.9</td>
</tr>
<tr>
<td>Parietal</td>
<td>17</td>
<td>16.2</td>
</tr>
<tr>
<td>Temporal</td>
<td>24</td>
<td>22.9</td>
</tr>
<tr>
<td>Occipital</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>Fronto-Parietal</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Fronto-Temporal</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Temporo-Parietal</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>Parieto-Occipital</td>
<td>5</td>
<td>4.8</td>
</tr>
<tr>
<td>Brainstem</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Multifocal</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Temporal + Thalamic</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Temporo-Insular</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Thalamic + Mid-Brain</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>105</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

41% of tumours were located in the frontal lobe, while tumours located in the temporal and parietal lobe accounted for 22% and 16%, respectively (Table 3.7). Overall, the right frontal lobe was the commonest site for intra-cranial tumours in the Irish cohort.
3.3.5: Performance Status

Table 3.8: Pre-operative Performance Status (Grade 3/4 Astrocytomas)

<table>
<thead>
<tr>
<th>Pre-op ECOG</th>
<th>Frequency</th>
<th>Percent</th>
<th>Valid Percent</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECOG 0</td>
<td>29</td>
<td>29.6</td>
<td>34.9</td>
<td>34.9</td>
</tr>
<tr>
<td>ECOG 1</td>
<td>45</td>
<td>45.9</td>
<td>54.2</td>
<td>89.2</td>
</tr>
<tr>
<td>ECOG 2</td>
<td>6</td>
<td>6.1</td>
<td>7.2</td>
<td>96.4</td>
</tr>
<tr>
<td>ECOG 3</td>
<td>2</td>
<td>2.0</td>
<td>2.4</td>
<td>98.8</td>
</tr>
<tr>
<td>ECOG 4</td>
<td>1</td>
<td>1.0</td>
<td>1.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>84.7</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

Unknown | 15 | 15.3 |
Total  | 98 | 100.0 |

More than 80% of the patients treated with bevacizumab had a good performance status (ECOG ≤ 2). Good performance status is an important factor in patient selection and has been shown to be of prognostic benefit in glioblastoma treatment.

3.3.6: Resection Rates

Table 3.8: Resection/5-ALA Correlation (Grade III/IV Astrocytoma)

<table>
<thead>
<tr>
<th>Resection</th>
<th>5-ALA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Total (GTR)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>NTR (&gt;95%)</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>80-95%</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>50-80%</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>51</td>
</tr>
</tbody>
</table>
The use of 5-ALA during surgery for Grade III and IV astrocytic tumours was not significant overall in predicting the EOR (p = 0.116). However, within this cohort, we observed that the use of 5-ALA achieves >50% resection (p<0.05).

3.3.7: Molecular Analysis

Table 3.9: IDH-1 Mutation in Astrocytomas

<table>
<thead>
<tr>
<th>IDH MUT</th>
<th>Astrocytic Grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 3</td>
<td>Grade 4</td>
</tr>
<tr>
<td>Mutation</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>7</td>
<td>46</td>
</tr>
<tr>
<td>Not Performed</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>N/A</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Pending</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>81</td>
</tr>
</tbody>
</table>

Immunohistochemistry revealed that IDH wild-type accounted for 57% of astrocytomas treated with bevacizumab in Beaumont Hospital. IDH mutated astrocytoma’s were found in 5% of cases and IDH mutational status was unknown in 19% of cases (Table 3.9/Figure 3.8).
Figure 3.8: IDH-1 Mutation in Astrocytomas

Table 3.10: IDH-1 Mutation in Glioblastoma (Age Distribution)

<table>
<thead>
<tr>
<th>IDH MUT</th>
<th>Mutation</th>
<th>Wild-Type</th>
<th>Not Performed</th>
<th>Unknown</th>
<th>N/A</th>
<th>Pending</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 18</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18 to 44</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>45 to 70</td>
<td>1</td>
<td>39</td>
<td>3</td>
<td>13</td>
<td>8</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>Over 70</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>56</td>
<td>6</td>
<td>19</td>
<td>10</td>
<td>2</td>
<td>98</td>
</tr>
</tbody>
</table>
69% of patients aged between 45-70 years were IDH Wild-type while younger patients (18-44 years) were 4 times more likely to have an IDH mutation (Table 3.10).

**Table 3.11: MGMT Methylation in Astrocytomas**

<table>
<thead>
<tr>
<th></th>
<th>Astrocytic Grade</th>
<th>Rad: GBM, Path: Grade 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 3</td>
<td>Grade 4</td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>3</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>2</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>Pending</td>
<td>1</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Not Performed</td>
<td>4</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>N/A</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>81</td>
<td>3</td>
</tr>
</tbody>
</table>

Methylation-specific PCR assays revealed MGMT methylation occurred in 27% of glioblastomas while 40% were unmethylated (Table 3.11/Figure 3.9). The methylation status was pending in 9 cases at the time of the analysis and was unknown in 8 cases. The prevalence of MGMT methylated glioblastoma is reported in up to 40% of patients\textsuperscript{16}. 

---

\textsuperscript{16}
Figure 3.9: MGMT Methylation in Astrocytomas

Table 3.12: MGMT Methylation in Astrocytomas (Age Distribution)

<table>
<thead>
<tr>
<th>MGMT</th>
<th>Methylated</th>
<th>Unmethylated</th>
<th>Pending</th>
<th>Not Performed</th>
<th>Unknown</th>
<th>N/A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18 to 44</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>45 to 70</td>
<td>16</td>
<td>26</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Over 70</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>36</td>
<td>10</td>
<td>15</td>
<td>8</td>
<td>4</td>
<td>98</td>
</tr>
</tbody>
</table>

No difference was seen in methylation status amongst different age groups (Table 3.11)
3.3.8: Bevacizumab Therapy (Grade III/IV)

Table 3.13: Bevacizumab Number of Cycles

<table>
<thead>
<tr>
<th>Cycle no.</th>
<th>N</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>9.79</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>6.50</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>60</td>
</tr>
</tbody>
</table>

The median number of cycles of bevacizumab for grade III/IV astrocytomas was 9.76 (Table 3.13/Figure 3.10) over a median of 3 months (Table 3.14/Figure 3.10).

Table 3.14: Bevacizumab Duration of Therapy

<table>
<thead>
<tr>
<th>Duration of Tx (Months)</th>
<th>N</th>
<th>98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>5.0918</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>3.0000</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>.00</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>29.00</td>
</tr>
</tbody>
</table>
Figure 3.10: Bevacizumab duration of therapy

Table 3.15: Time Interval from First Surgery to Start of Bevacizumab

<table>
<thead>
<tr>
<th>First Surgery to Start of Avastin (Months)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Known</td>
<td>94</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th>15.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>12.00</td>
</tr>
<tr>
<td>Minimum</td>
<td>4</td>
</tr>
<tr>
<td>Maximum</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 3.11: Time Interval from First Surgery to Start of Bevacizuamb Therapy

Table 3.16: Time Interval from Surgery to Mortality

<table>
<thead>
<tr>
<th>First Surgery to Death</th>
<th>Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valid</td>
<td>69</td>
</tr>
<tr>
<td>Unknown</td>
<td>29</td>
</tr>
<tr>
<td>Mean</td>
<td>21.07</td>
</tr>
<tr>
<td>Median</td>
<td>18.00</td>
</tr>
<tr>
<td>Minimum</td>
<td>7</td>
</tr>
<tr>
<td>Maximum</td>
<td>59</td>
</tr>
</tbody>
</table>
Figure 3.12: Time Interval from Surgery to Mortality

The interval time from the first surgery to the start of bevacizumab was 12 months (Table 3.15/Figure 3.11) with a survival time of 18 months (Table 3.16/Figure 3.12).
3.3.9: Overall Survival

Table 3.17: Tumour grade/Mortality Correlation

<table>
<thead>
<tr>
<th>WHO GRADE</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO Grade 3</td>
<td>10</td>
<td>27.20</td>
<td>27.00</td>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td>WHO Grade 4</td>
<td>58</td>
<td>20.09</td>
<td>18.00</td>
<td>7</td>
<td>59</td>
</tr>
<tr>
<td>Rad Grade 4, Histo Grade 3</td>
<td>1</td>
<td>17.00</td>
<td>17.00</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>21.07</td>
<td>18.00</td>
<td>7</td>
<td>59</td>
</tr>
</tbody>
</table>

Overall survival in the Irish cohort of glioblastoma and grade III astrocytoma, including the administration of bevacizumab was 18 months and 27 months, respectively (Table 3.17). The single case of histological/radiological mismatch, survival was 17 months. This likely represented a sampling error and supports the rationale for treating these patients as a grade IV.

Table 3.18: Tumour Grade/Mortality Correlation

<table>
<thead>
<tr>
<th>WHO GRADE</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of Bevacizumab to Death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO Grade 3</td>
<td>12</td>
<td>5.00</td>
<td>3.00</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>WHO Grade 4</td>
<td>57</td>
<td>6.86</td>
<td>5.00</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>Rad Grade 4, Histo Grade 3</td>
<td>1</td>
<td>8.00</td>
<td>8.00</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>6.56</td>
<td>5.00</td>
<td>1</td>
<td>22</td>
</tr>
</tbody>
</table>

The administration of bevacizumab improved the survival of glioblastoma recurrence and grade III astrocytoma’s by 5 months and 3 months, respectively (Table 3.18). This might be explained by the last line salvage approach in deteriorating grade III patients refractory to alkylating agents.
Figure 3.13: Commencement of Bevacizumab to Mortality (months)
### Table 3.19: Glioblastoma Data

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Grade 4 Patients (n = 83)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
</tr>
<tr>
<td>Age, Median (range)</td>
<td>57 (25-84)</td>
</tr>
<tr>
<td>Age Groups (n)</td>
<td></td>
</tr>
<tr>
<td>18 – 44</td>
<td>21.7% (18)</td>
</tr>
<tr>
<td>45 – 70</td>
<td>69.9% (58)</td>
</tr>
<tr>
<td>Over 70</td>
<td>8.4% (7)</td>
</tr>
<tr>
<td>Female (n)</td>
<td>32.5% (27)</td>
</tr>
<tr>
<td><strong>MGMT Status (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>26.5% (22)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>41% (34)</td>
</tr>
<tr>
<td>Unknown</td>
<td>32.5% (27)</td>
</tr>
<tr>
<td><strong>IDH1 Mutation Status (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>6% (5)</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>57.8% (48)</td>
</tr>
<tr>
<td>Unknown</td>
<td>36.1% (30)</td>
</tr>
<tr>
<td><strong>Tumour Location</strong></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>42.2% (35)</td>
</tr>
<tr>
<td>Non-Frontal</td>
<td>57.8% (48)</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td></td>
</tr>
<tr>
<td>No of Resections, Median (range)</td>
<td>1.00 (1 – 3)</td>
</tr>
<tr>
<td>Patients with one resection (n)</td>
<td>77.1% (64)</td>
</tr>
<tr>
<td>Patients with two resections (n)</td>
<td>12% (10)</td>
</tr>
<tr>
<td>Patients with three resections (n)</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td>Patients with Biopsy only (n)</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td><strong>5-ALA Used (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20.5% (17)</td>
</tr>
<tr>
<td>No</td>
<td>73.5% (61)</td>
</tr>
<tr>
<td>N/A</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2.4% (2)</td>
</tr>
<tr>
<td><strong>Extent of Resection (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20.5% (17)</td>
</tr>
<tr>
<td>Near Total</td>
<td>9.6% (8)</td>
</tr>
<tr>
<td>80 – 95%</td>
<td>21.7% (18)</td>
</tr>
<tr>
<td>50 – 80%</td>
<td>20.5% (17)</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>20.5% (17)</td>
</tr>
<tr>
<td>N/A</td>
<td>3.6% (3)</td>
</tr>
<tr>
<td>Pre-Op ECOG, Median (Range)</td>
<td>1 (0 – 4)</td>
</tr>
<tr>
<td>Post-Op, Median (Range)</td>
<td>1 (0 – 3)</td>
</tr>
<tr>
<td>Time from first surgery to Bevacizumab</td>
<td></td>
</tr>
<tr>
<td>Months, median (range)</td>
<td>12 (4 – 55)</td>
</tr>
</tbody>
</table>
Specific to glioblastoma treated with bevacizumab, the median age profile of patients was 57 years of age, with 70% of patients aged between 45 and 70 years old. Within this cohort 67.5% of the patients were male with just under half of the tumours located in the frontal lobe. In total, 26.5% of the tumours were found to be methylated with only 6% IDH mutated, suggesting that these tumours were characteristic of a primary glioblastoma. Over two-thirds of the patients had one operation, while thirteen patients had more than one operation. During surgery, 5-ALA was used in only 20% of cases and over 30% of the patients had a total/near total resection. Overall the patients were of good performance status in the peri-operative phase. The median wait time from surgery to the commencement of bevacizumab was 12 months.

### Table 3.20: Glioblastoma Univariate Analysis (Bevacizumab to Death)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard Ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Female vs Male)</td>
<td>0.81 (0.47 – 1.4)</td>
<td>0.45</td>
</tr>
<tr>
<td>Age (&lt;55 vs ≥55)</td>
<td>1.08 (0.64 – 1.83)</td>
<td>0.767</td>
</tr>
<tr>
<td>MGMT status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated vs Un-methylated</td>
<td>1.68 (0.86 – 3.29)</td>
<td>0.129</td>
</tr>
<tr>
<td>IDH Mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant vs Wild-Type</td>
<td>1.4 (0.49 – 3.98)</td>
<td>0.53</td>
</tr>
<tr>
<td>Frontal vs Non Frontal</td>
<td>1.31 (0.765 – 2.23)</td>
<td>0.33</td>
</tr>
<tr>
<td>Extent of Resection (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total vs Near Total</td>
<td>1.23 (0.45 – 3.34)</td>
<td>0.68</td>
</tr>
<tr>
<td>Total vs 80 – 95%</td>
<td>1.43 (0.63 – 3.25)</td>
<td>0.4</td>
</tr>
<tr>
<td>Total vs 50 – 80%</td>
<td>1.55 (0.69 – 3.49)</td>
<td>0.29</td>
</tr>
<tr>
<td>Total vs &lt;50%</td>
<td>3.54 (0.96 – 13.03)</td>
<td>0.06</td>
</tr>
<tr>
<td>No. of Chemotherapy (1 vs ≥2)</td>
<td>0.94 (0.4 – 2.21)</td>
<td>0.89</td>
</tr>
<tr>
<td>No. of Radiotherapy (1 vs ≥2)</td>
<td>0.94 (0.4 – 2.21)</td>
<td>0.89</td>
</tr>
<tr>
<td>Pre-Op ECOG</td>
<td>1.245 (0.89 – 1.742)</td>
<td>0.20</td>
</tr>
<tr>
<td>Post-Op ECOG</td>
<td>1.37 (0.93 – 2.019)</td>
<td>0.11</td>
</tr>
</tbody>
</table>
As shown in Figure 3.14, patients over the age of 55 years had a better outcome, although this was non significant (p= 0.76).

Figure 3.15: Kaplan - Meier Survival Estimates of Overall Survival according to IDH Mutational Status.
Similarly, in Figure 3.15, patients with the favourable prognostic IDH mutation had a longer survival, however it was non significant (p=0.53)

Figure 3.16: Kaplan-Meier Survival Estimates of Overall Survival according to MGMT Status.

Patients found to have MGMT methylation also had a non significant survival benefit compared to unmethylated (p=0.129), as shown in Figure 3.16

Figure 3.17: Kaplan-Meier Survival Estimates of Overall Survival according to Resection Margin.
Patients who underwent a gross total resection/near total resection survived longer compared to those who have smaller resection rates. There was no significant difference in survival when comparing gross total resection and near total resection (p=0.68). Patients who had only <50% resection had the poorest outcome, as demonstrated in Figure 3.17.

Figure 3.18: Kaplan-Meier Survival Estimates of Overall Survival according to Location.

Figure 3.18 demonstrated the marginal non-significant survival benefit in patients whose glioblastoma was located in the frontal lobe (p=0.33).
Table 3.21: Glioblastoma Univariate Analysis (First Surgery to Death)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard Ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Female vs Male)</td>
<td>0.76 (0.44 – 1.33)</td>
<td>0.76</td>
</tr>
<tr>
<td>Age (&lt;55 vs ≥55)</td>
<td>1.28 (0.76 – 2.17)</td>
<td>0.353</td>
</tr>
<tr>
<td>MGMT status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated vs Un-methylated</td>
<td>2.76 (1.38 – 5.525)</td>
<td>0.004</td>
</tr>
<tr>
<td>IDH Mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant vs Wild-Type</td>
<td>1.61 (0.56 – 4.6)</td>
<td>0.373</td>
</tr>
<tr>
<td>Frontal vs Non-Frontal</td>
<td>1.29 (0.75 – 2.196)</td>
<td>0.355</td>
</tr>
<tr>
<td>Extent of Resection (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total vs Near Total</td>
<td>1.65 (0.6 – 4.56)</td>
<td>0.33</td>
</tr>
<tr>
<td>Total vs 80 – 95%</td>
<td>1.94 (0.85 – 4.43)</td>
<td>0.12</td>
</tr>
<tr>
<td>Total vs 50 – 80%</td>
<td>2.46 (1.06 – 5.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>Total vs &lt;50%</td>
<td>2.06 (0.56 – 7.54)</td>
<td>0.28</td>
</tr>
<tr>
<td>No. of Chemotherapy (1 vs ≥2)</td>
<td>0.52 (0.21 – 1.3)</td>
<td>0.16</td>
</tr>
<tr>
<td>No. of Radiotherapy (1 vs ≥2)</td>
<td>1.93 (0.77 – 4.86)</td>
<td>0.16</td>
</tr>
<tr>
<td>Pre-Op ECOG</td>
<td>1.25 (0.91 – 1.71)</td>
<td>0.178</td>
</tr>
<tr>
<td>Post-Op ECOG</td>
<td>1.26 (0.87 – 1.83)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Figure 3.20: Kaplan - Meier Survival Estimates of Overall Survival according to Age
When examining the univariate analysis in glioblastoma patients from the time of surgery to death (Figure 3.20), we found no difference in outcome according to age (p=0.353).

**Figure 3.21: Kaplan-Meier Survival Estimates of Overall Survival according to MGMT status.**

A significant survival benefit was demonstrated in Figure 3.21 when comparing methylated versus unmethylated glioblastomas. Patients with a positive methylation status lived longer compared to the unmethylated group (p=0.004).

**Figure 3.22: Kaplan-Meier Survival Estimates of Overall Survival according to Resection Margin.**
Patients who underwent a gross total resection/near total resection survived longer compared to those who had small resection rates. There was no significant difference in survival when comparing gross total resection and near total resection (p=0.33). However, there was a significant survival benefit when comparing gross total section to patients who had a 50-80% resection (p=0.04)
### Table 3.22: Secondary Glioblastoma Demographics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Grade 4 Patients (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
</tr>
<tr>
<td>Age, Median (range)</td>
<td>58 (28-84)</td>
</tr>
<tr>
<td>Age Groups (n)</td>
<td></td>
</tr>
<tr>
<td>18 – 44</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td>45 – 70</td>
<td>72.7% (8)</td>
</tr>
<tr>
<td>Female (n)</td>
<td>45.5% (5)</td>
</tr>
<tr>
<td><strong>MGMT Status (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>54.5% (6)</td>
</tr>
<tr>
<td>Unknown</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td><strong>IDH1 Mutation Status (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td>Absent</td>
<td>54.5% (6)</td>
</tr>
<tr>
<td>Unknown</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td><strong>Tumour Location</strong></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>36.4% (4)</td>
</tr>
<tr>
<td>Non-Frontal</td>
<td>63.6% (7)</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td></td>
</tr>
<tr>
<td>No of Resections, Median (range)</td>
<td>2.00 (1 – 3)</td>
</tr>
<tr>
<td>Patients with one resection (n)</td>
<td>36.4% (4)</td>
</tr>
<tr>
<td>Patients with two resections (n)</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td>Patients with three resections (n)</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td><strong>5-ALA Used (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td>No</td>
<td>81.8% (9)</td>
</tr>
<tr>
<td><strong>Extent of Resection (n)</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td>Near Total</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td>80 – 95%</td>
<td>18.2% (2)</td>
</tr>
<tr>
<td>50 – 80%</td>
<td>9.1% (1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>27.3% (3)</td>
</tr>
<tr>
<td><strong>Pre-Op ECOG, Median (Range)</strong></td>
<td>1 (0 – 1)</td>
</tr>
<tr>
<td><strong>Post-Op ECOG, Median (Range)</strong></td>
<td>1 (0 – 2)</td>
</tr>
<tr>
<td><strong>Time from first surgery to Bevacizumab</strong></td>
<td></td>
</tr>
<tr>
<td>Months, median (range)</td>
<td>9 (4 – 29)</td>
</tr>
</tbody>
</table>
Specific to "secondary "glioblastoma (glioblastoma that originated from a lower grade or tumours with IDH mutation) treated with bevacizumab, the median age profile of patients was 58 years of age, with 72% of patients aged between 45 and 70 years old. Within this cohort 54.5% of the patients were male with 36.4% of the tumours located in the frontal lobe. In total, 18.2% of the tumours were found to be methylated. However a much higher percentage were found to have to be IDH mutated (27.3%), characteristic of a secondary glioblastoma.

The median number of operations in this cohort was 2 procedures, with over half of the patients having more than 1 operation. This reflects the natural history of a “secondary” glioblastoma relating to the malignant progression from a lower grade glioma. Overall the patients were of good performance status in the peri-operative phase. The median wait time from surgery to the commencement of bevacizumab was 9 months.

Table 3.23 Secondary Glioblastoma Univariate Analysis (Bevacizumab to Death)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard Ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate Analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Female vs Male)</td>
<td>0.29 (0.07 – 1.26)</td>
<td>0.098</td>
</tr>
<tr>
<td>Age (&lt;55 vs ≥55)</td>
<td>1.178 (0.28 – 4.99)</td>
<td>0.83</td>
</tr>
<tr>
<td>MGMT status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated vs Un-methylated</td>
<td>62.75 (0.03 – 119685.85)</td>
<td>0.283</td>
</tr>
<tr>
<td>IDH Mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present vs Absent</td>
<td>2.41 (0.45 – 12.76)</td>
<td>0.302</td>
</tr>
<tr>
<td>Frontal vs Non-Frontal</td>
<td>3.00 (0.555-16.249)</td>
<td>0.202</td>
</tr>
<tr>
<td>Extent of Resection (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total vs Near Total</td>
<td>0.36 (0.029 – 4.38)</td>
<td>0.421</td>
</tr>
<tr>
<td>Total vs 80 – 95%</td>
<td>1.07 (0.12 – 9.51)</td>
<td>0.953</td>
</tr>
<tr>
<td>Total vs 50 – 80%</td>
<td>2.35 (0.18 – 30.61)</td>
<td>0.51</td>
</tr>
<tr>
<td>No. of Chemotherapy (1 vs ≥2)</td>
<td>0.531 (0.06 – 4.79)</td>
<td>0.573</td>
</tr>
<tr>
<td>No. of Radiotherapy (1 vs ≥2)</td>
<td>1.88 (0.21 – 16.98)</td>
<td>0.573</td>
</tr>
<tr>
<td>Pre-Op ECOG</td>
<td>0.46 (0.08 – 2.78)</td>
<td>0.397</td>
</tr>
<tr>
<td>Post-Op ECOG</td>
<td>1.46 (0.51 – 4.18)</td>
<td>0.486</td>
</tr>
</tbody>
</table>
As shown in Figure 3.23, patients over the age of 55 years had a better outcome, although this was non significant ($p=0.83$).

Similarly, in Figure 3.24, patients with the favourable prognostic IDH mutation had a longer survival, however it was non significant ($p=0.302$)
Figure 3.25: Kaplan-Meier Survival Estimates of Overall Survival according to MGMT Status.

Patients found to have MGMT methylation also had a non-significant survival benefit compared to unmethylated ($p=0.283$), as shown in Figure 3.25.

Figure 3.26: Kaplan-Meier Survival Estimates of Overall Survival according to Location.

Once more, figure 3.26 demonstrated the non-significant survival benefit in patients whose glioblastoma was located in the frontal lobe ($p=0.202$).
Table 3.24: Secondary Glioblastoma Univariate Analysis (First Surgery to Death)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard Ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Female vs Male)</td>
<td>0.27 (0.051 – 1.4)</td>
<td>0.118</td>
</tr>
<tr>
<td>Age (&lt;55 vs ≥55)</td>
<td>0.775 (0.18 – 3.39)</td>
<td>0.74</td>
</tr>
<tr>
<td>MGMT status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated vs Un-methylated</td>
<td>49.89 (0.31 – 91848.3)</td>
<td>0.31</td>
</tr>
<tr>
<td>IDH Mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present vs Absent</td>
<td>91.94 (0.06 – 148157.5)</td>
<td>0.23</td>
</tr>
<tr>
<td>Frontal v Non-Frontal</td>
<td>4.01 (0.72-22.21)</td>
<td>0.11</td>
</tr>
<tr>
<td>Extent of Resection (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total vs Near Total</td>
<td>0.000 (0.00 –0.000)</td>
<td>0.96</td>
</tr>
<tr>
<td>Total vs 80 – 95%</td>
<td>2.45 (0.299 – 20.062)</td>
<td>0.404</td>
</tr>
<tr>
<td>Total vs 50 – 80%</td>
<td>0.79 (0.069 – 9.185)</td>
<td>0.854</td>
</tr>
<tr>
<td>No. of Chemotherapy (1 vs ≥2)</td>
<td>1.11 (0.13 – 9.36)</td>
<td>0.927</td>
</tr>
<tr>
<td>No. of Radiotherapy (1 vs ≥2)</td>
<td>0.91 (0.11 – 7.67)</td>
<td>0.927</td>
</tr>
<tr>
<td>Pre-Op ECOG</td>
<td>0.89 (0.18 – 4.44)</td>
<td>0.888</td>
</tr>
<tr>
<td>Post-Op ECOG</td>
<td>1.12 (0.47 – 2.68)</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Figure 3.27: Kaplan - Meier Survival Estimates of Overall Survival according to Age.
When examining the univariate analysis in “secondary” glioblastoma patients from the time of surgery to death (Figure 3.27), we found no difference in outcome according to age (p=0.118).

Figure 3.28: Kaplan-Meier Survival Estimates of Overall Survival according to IDH Mutational Status.

The importance of IDH mutation in “secondary” glioblastoma was highlighted in Figure 3.28. IDH mutated patients had a longer survival compared to IDH wild-type, however it was non significant (p=0.23)

Figure 3.29: Kaplan-Meier Survival Estimates of Overall Survival according to MGMT Status.
A significant survival benefit was demonstrated in Figure 3.29 when comparing methylated versus unmethylated glioblastomas. Patients with a positive methylation status lived longer compared to the unmethylated group (p=0.31).

![Kaplan-Meier Survival Estimates of Overall Survival according to Location.](image)

**Figure 3.30: Kaplan-Meier Survival Estimates of Overall Survival according to Location.**

Once more, figure 3.30 demonstrated the non significant survival benefit in patients whose glioblastoma was located in the frontal lobe (p=0.11).
3.4 Discussion

This retrospective study revealed that 105 patients with intra-cranial tumours were treated with bevacizumab in the Irish National Neurosurgical Center over a 3 year period. Over 91% of the patients were treated for an astrocytoma of which 77% were histologically diagnosed glioblastoma recurrence. Glioblastoma were more common in men, >45 years of age and located in the right cerebral hemisphere with 41.9% occurring in the frontal lobe. Literature examining the use of bevacizumab outside the strict criteria of clinical trials is lacking. Our study represents real life data demonstrating the clinical efficacy/prognostic factors in bevacizumab treated patients in glioblastoma recurrence.

The Irish cohort of recurrent glioblastoma had a median survival of 5 months which is comparable to other published studies in similar populations\(^{27,31,20,41}\) while the overall survival from the time of surgery was 18 months, compared to 27 months for grade III astrocytomas. Bevacizumab was commenced 12 months after the initial surgery with a median of 3 months treatment duration.

MGMT methylation, frontal tumour, EOR and IDH-mutation were shown to be independent positive prognostic factors for overall survival in bevacizumab treated patients in recurrent glioblastoma. The importance of MGMT methylation as a prognostic biomarker was also replicated in the Irish population, particularly when examining survival from the time of the initial surgery to death. Conversely, the effect of MGMT methylation was not as pronounced when assessing the outcome from the start of bevacizumab to death. Our results corroborate the findings of Schaub et al\(^{304}\) but do not correspond to the Phase II BELOB study\(^{302}\), in which some patients received lomustine (alkylating agent) following bevacizumab failure, thereby conveying a favourable response rate in MGMT methylated patients.
While the difference in other parameters was not significant in this study, there was a favourable trend to show an extended survival benefit. The added benefits of a favourable tumour location in the frontal lobe was also seen in Irish patients. It remains to be determined whether this survival benefit is a reflection of a less invasive biological phenotype of frontal glioblastoma or that these tumours are more amenable to complete resection. The use of 5-ALA during surgery for Grade III and IV astrocytic tumours was not significant overall in predicting amount of resection achieved (p = 0.116). However, within this cohort, we observed that the use of 5-ALA achieved >50% resection (p<0.05). The paucity of 5-ALA utilization in our unit (20% of cases) reflected its introduction as a surgical adjunct in late 2012.

Interestingly, no difference in survival was observed between those aged <55 years of age and >55 years of age, supporting in the rationale for treating elderly patients with bevacizumab. Consequently, the University of Zurich are conducting the ARTE trial (Avastin Plus Radiotherapy in Elderly Patients with Glioblastoma- NCT01443676) and Genetech Inc are also assessing bevacizumab response rates in elderly patients (NCT01149850).

Within our cohort, the majority of our patients were of good performance status (>80%, ECOG≤2) thereby generating a selection bias. However, the clinical benefit of treating patients with a poor performance status is minimal and careful patient selection is warranted.

Univariate analysis in glioblastoma cases that progressed from a lower grade astrocytoma (secondary glioblastoma) showed that the median age of this cohort was similar to the “all glioblastoma” group, although a larger percentage of patients were in the 18-44 years of age group (27.3%). In addition, more patients were IDH-mutated supporting the hypothesis that this genetic alteration is a surrogate of secondary glioblastoma. The prognostic survival benefit of IDH mutation in secondary glioblastoma was also demonstrated in our study.
Interestingly, we found a favourable survival trend in secondary glioblastoma located in the frontal lobe, although this was not significant. Padlor et al, recently suggested that the benefit of IDH mutations arises from different locations and its prognostic effect is lost once controlled for location\textsuperscript{306}. It is well known that periventricular/corpus callosum tumours have a poorer outcome compared to tumours outside of these areas\textsuperscript{307-309}. It remains to be established if tumour location is surrogate marker of the biological activity of the tumour.

We acknowledge that the sample size in the study is small and therefore underpower the statistical analysis. The small sample size reflect the judicious use of bevacizumab in this country and difficulties in follow up with the patients due to the geographical distribution of the cohort. However our results corroborate other groups findings and warrants further investigations.

In summary, this single site study demonstrates the importance of tumour location, MGMT methylation, IDH and EOR as prognostic factors in recurrent glioblastoma. Although the study is retrospective, the overall survival in Irish patients is comparable to international standards. Our findings also support the use of bevacizumab in elderly patients of good performance status.

Despite the apparent survival benefit in non randomized clinical studies, phase III trials have not demonstrated any overall survival benefit, the explanation of which can be attributed to the ability of the tumour to resist the anti-angiogenic inhibition via activation of aberrant pathways. Therefore, we hypothesized that combining bevacizumab with an mTOR/PI3K inhibitor would enhance the anti-tumour/anti-angiogenic effects of bevacizumab in an orthotopic model.
Toxicity & Survival:

Response to a combined Novel Therapeutic Strategy
4.1 Introduction

Glioblastoma is the most aggressive malignant primary brain tumor in adults (median age, 64 y) with a preponderance in men (1.3 – 1.6:1), whites, and those of European descent (2:1 compared with African Americans)⁷. The annual incidence ranges from 3 to 5 newly diagnosed cases per 100 000 population. Therapeutic advances over the last decade have led to improvements in both patients’ life expectancy and quality of life.

These tumours are highly angiogenic, therefore generate a convincing rationale for targeting the tumor vasculature. High-grade gliomas secrete large amounts of VEGF, which initiates a cascade of downstream intracellular signal transduction pathways resulting in endothelial cell proliferation and migration, vascular permeability and subsequently the formation of new blood vessels³¹⁰. Inhibiting VEGF is an effective anticancer therapy and several clinical trials have demonstrated a high response rate and improvements in progression-free survival rate in patients with recurrent glioblastoma treated with bevacizumab, with or without Irinotecan²³⁹,³¹¹, compared with outcomes for historical controls¹⁶². Despite an improved PFS-6, there is no current data illustrating an improved overall survival in glioblastoma patients treated with bevacizumab.

The use of tyrosine kinase inhibitors in glioblastoma clinical trials have also been largely disappointing. Cediranib (AZD2171, Recentin™, a small molecule, orally bioavailable tyrosine kinase inhibitor (TKI) of VEGFR2, PDGF, receptor-α and –β, and stem growth factor receptor (c-Kit)³¹² and cilingitide (EMD121974, a cyclic RGD-mimetic peptide selective inhibitor of both αvβ3 and αvβ5 integrins) has been investigated in the treatment of recurrent GBM, but failed to prolong survival in a phase III randomised trial. Other more conventional, multi-targeted TKIs, such as sunitinib and sorafenib, (which inhibit activation of downstream VEGF receptor (VEGFR-2), signalling pathways as well as targeting other pathways involved in angiogenesis including PDGFR, fms-related tyrosine kinase 3 (FLT3), c-KIT³¹³ showed pre-clinical promise for
application in GBM settings. To date there is lack of available clinical data demonstrating significant benefit of sunitinib and sorafenib in glioblastoma. In preclinical studies, the combination of VEGF and mTOR inhibitors has demonstrated additive antitumour effects in multiple in vivo models, including hepatocellular carcinoma, renal cell carcinoma, ovarian cancer and pancreatic cancer.

However, inhibition of multiple targets increases the likelihood of drug toxicity since normal tissue may also express the targeted molecules. Multiple target proteins also make the intercellular effects more unpredictable. Several clinical trials suggest that combinations of molecularly targeted anticancer agents (MTA) can lead to enhanced toxicities and that dose reduction is often required for regimens to be tolerable. Sunitinib, which inhibits a broad range of receptor tyrosine kinases, including vEGFR, has been tested in two Phase I trials combined with bevacizumab. This combination was associated with considerably increased rates of grade 3 to grade 4 side effects such as hypertension, thrombocytopenia and proteinuria, even though the determination of the MTD, which was based on observations in the first cycle of treatment, suggested that full doses of both agents could be administered.

However, prolonged therapy with this regimen in patients with renal cell carcinoma (RCC) showed an unexpectedly high incidence of thrombotic microangiopathy, with presentations ranging from subclinical findings to thrombocytopenia or to acute renal failure. Several patients also developed a constellation of hypertensive crisis, reversible posterior leukoencephalopathy, and nephrotic syndrome. Furthermore, in a phase I trial of bevacizumab and everolimus, full doses of both agents were tolerable in the combination. However, it was noted in the Phase II evaluation of these agents that when more patients were treated for longer durations, grade 3 to grade 4 proteinuria was observed in 26% of the patients, a much higher rate than the 7% proteinuria rate observed with bevacizumab alone in patients with RCC.
4.2 Chapter Aims

The metabolic effects of combining a VEGF inhibitor and a dual PI3K/mTOR inhibitor has not being previously examined in the literature. The aim of this study was to examine the tolerability and dosing regime, in vivo, of a novel drug therapy combining bevacizumab and a dual PI3K/mTOR inhibitor (NVP-BEZ235). Subsequently, overall survival was examined in orthotopic explant models using the novel therapeutic drug strategy.
4.3 Results

4.3.1 Toxicity I

In order to assess the efficacy of bevacizumab in combination with a dual PI3K/mTOR inhibitor in an orthotopic mouse model, we first performed a toxicity study to examine for unwanted toxic side effects of the combination therapy. This experiment was carried out using an incremental dosing regime.

**Figure 4.1: Animal Weights: Toxicity I**

Overall, the mice tolerated the drug combination. This diagram represents the individual mice and their respective weights. Of note, mouse 2 had a sharp decline in weight initially (<20%) which reflected a substandard oral gavage at the beginning of the study (day 2). Other animal parameters (behaviour, skin, breathing) were also within normal limits during the study.
Figure 4.2: Mean Animal Weights: Toxicity I (Mean ± SD)

The dosing regime was well tolerated over the 3 week period and had no adverse reactions. This diagram demonstrates animal weights were maintained throughout the study. Other animal parameters (behaviour, skin, breathing) were also within normal limits during the study.

4.3.2 Toxicity II

Following successful completion of the initial lower drug combination, an increased dose regime was implemented.
Overall, the mice tolerated the higher drug dose combination. This diagram represents the individual mice and their respective weights. Of note, mouse 2 had a sharp decline in weight initially (<20%) which reflected a substandard oral gavage at the beginning of the study (day 4). Other animal parameters (behaviour, skin, breathing) were also within normal limits during the study.
Figure 4.4: Mean Animal Weights: Toxicity II (Mean ± SD)

The higher dosing regime was well tolerated over the 3 week period and had no adverse reactions. This diagram demonstrates that animal weights were maintained throughout the study. Other animal parameters (behaviour, skin, breathing) were also within normal limits during the study.

4.3.3 Overall Survival

21 days post implantation of U87MG-luc2 cells, mice were randomized into 4 groups:

Group 1. Vehicle control
Group 2. BEZ 235 (45mg/kg po od, 5/7, 2/7 off)
Group 3. Bevacizumab (10mg/kg ip od, alternating days, 12/7)
Group 4. BEZ 235 & Bevacizumab (45mg/kg po od, 5/7, 2/7 off & 10mg/kg ip od, alternating days, 12/7)
Figure 4.5: Overall Survival

Kaplan Meier analysis demonstrated a median survival time of 39 days in the vehicle control group, with a statistically significant survival benefit in bevacizumab/combined treatment groups \((p = 0.0018 \; ; \; p = 0.0007, \) respectively ). There was no survival benefit in the BEZ 235 group \((p = 0.2490).\) No mice in the vehicle control group survived to the end of the study. There was no statistical difference between the treatment groups.
4.3.4 Animal Weights

A sharp decline in animal weights is observed in the control group from day 27. A transient reduction in animal weights is seen in the BEZ 235 monotherapy group from day 22 over a 6 day period. The bevacizumab monotherapy and combined therapy groups remained stable throughout the study. (mouse monitoring score sheets - Appendix I)
A sharp decline in animal weights is observed in the vehicle control group, corresponding to a reduction in overall survival. In the BEZ235 monotherapy group, 2 mice died before the end of treatment - 1 mouse died immediately post oral gavage (respiratory complications) on day 37 and the other mouse died on day 36, secondary to tumour burden. The bevacizumab and combined therapy groups remained stable, conveying a significant benefit in overall survival.
4.3.5 Histology

After the last BLI imaging session, mice were kept deeply anesthetized and perfused transcardially with saline for 5 min, followed by 4% PFA for 2 min. Brains were post-fixed in PFA overnight.

4.3.5.1 H&E

![Figure 4.8: H&E staining - representative animal per group (4x magnification: Scale 100μm).](image)

As demonstrated in Figure 4.8, overall tumour volumes were significantly larger in the untreated control group and BEZ235, while the combined group had smaller tumour volumes compared to bevacizumab monotherapy.
4.3.5.2 Ki67

Figure 4.9: Ki67 staining - representative animal per group (20x magnification: Scale 100μm).

Ki67 staining indicated that tumour cell proliferation was not further reduced by treatment with the BEV/BEZ235 combination than by treatment with BEV alone ($p = 0.1255$).
As demonstrated in Figure 4.10, the rate of proliferating cells was significantly reduced in the bevacizumab/combined treatment groups compared to the control group ($p = 0.0357$; $p = 0.0238$, respectively). We observed a similar trend in the bevacizumab/combined treatment groups versus BEZ235 ($p = 0.0357$; $p = 0.0238$, respectively).

4.3.5.3 vWF

![Figure 4.11: vWF staining- representative animal per group (10x magnification: Scale 100μm).](image)
As demonstrated in Figure 4.12, our treatment strategies had a profound antagonistic effect on the angiogenic properties of the tumours. The combined therapeutic approach significantly reduced the formation of new blood vessels compared to bevacizumab alone (\( p = 0.05 \)). Bevacizumab had a greater effect on reducing blood vessel formation than BEZ235 alone (\( p = 0.01 \)). While we observed a reduction in angiogenesis in the BEZ235 group versus control group, this was not significant.
Figure 4.13: vWF staining (vessel size) - representative animal per group (10x magnification: Scale 100μm).

Figure 4.14: Average Area per Vessel (%FOV: Mean ± SD).

As demonstrated in Figure 4.14, the average size of the vessels increased in the treatment groups. The combination/BEZ235 group produced significantly larger blood vessels than the control group (p = 0.025; p = 0.045, respectively)
4.3.5.4 TUNEL

<table>
<thead>
<tr>
<th>Control</th>
<th>BEZ 235</th>
<th>BEV</th>
<th>Combined</th>
</tr>
</thead>
</table>

Figure 4.15: TUNEL staining.

As demonstrated in Figure 4.15, minimal apoptotic staining was observed. Small quantities were present on a signal animal from the combined treatment group, while N=1 animal from the control group had a positive staining, relating to focal areas of necrosis (Figure 4.13). Therefore, TUNEL staining was not quantified in this study.

Figure 4.16: TUNEL staining-representative animal from control group.
4.4 Discussion

The efficacy of targeted monotherapy in glioblastoma have being largely disappointing. With an increased understanding of gliomagenesis, the emphasis has shifted to multi-target inhibition that either affects more than one pathway or the same pathway at more than one site. However, toxicity is often the rate limiting step with such an approach, predominately due to an incomplete understanding of the mechanistic action of the targeted therapy and lack of reproducible preclinical models.

While previous studies had successfully treated mice with BEZ235 in vivo\textsuperscript{244,245}, no studies have combined a PI3K/mTOR inhibitor and bevacizumab in a clinical or preclinical setting. Initially, we assessed the toxic effects of an oral PI3K/mTOR inhibitor in combination with bevacizumab and on successful completion we examined the overall survival benefit and histological parameters in a malignant glioma mouse model following the dual treatment strategy.

Overall, the toxicity study was tolerated well in vivo. No adverse effects were observed. Administration of BEZ235 via the challenging procedure of oral gavage, resulted in reductions of animal weights during (N=2) the toxicity study. Nevertheless, all other treated mice did not show any adverse side effects as illustrated in the mouse monitoring score sheets (Appendix I). Consequently, the higher drug concentrations were implemented in the efficacy study. Similarly, mice tolerated the drug combination throughout the treatment period. Oral gavage resulted in a direct mortality in 1 case. The mortality could be attributed to sub-optimal placement of the dosing catheter, which was appreciated immediately due to respiratory complications.
Kaplan Meier analysis demonstrated a statistically significant survival benefit in bevacizumab/combined treatment groups versus the control group (Figure 4.5), significance which was further heightened due to no mice in the control group alive at the study conclusion. There was no survival benefit in the BEZ235 group compared to control, results which differ from previous pre-clinical data. Although Liu et al, demonstrated that BEZ235 significantly prolonged the survival of intra-cranial tumour-bearing mice, treatment started day 4 after implantation\textsuperscript{245}. Therefore, one could argue that tumour growth may not be in the exponential growth phase and treatment was commenced prematurely. In a subsequent study, Maria et al, demonstrated the potent anti-tumour effects of BEZ235\textsuperscript{244}. This authors, utilized a subcutaneous glioma model which is not as clinically relevant as an intra-cranial glioma model, such as the one implemented in this study.

As illustrated in Figure 4.5, there was no significant difference in survival between the treatment groups, which could be explained by the censorship of the survival data. Histological analyses revealed significantly smaller tumours in the bevacizumab/combined treatment groups versus BEZ235 and control groups. Similarly, the amount of proliferating cells was reduced significantly in the bevacizumab/combined approach versus BEZ235 and control groups. No difference in proliferation was observed between BEZ235 and the vehicle control group. Quantification of vWF demonstrated significantly less blood vessels in the combined therapy group compared to beavacizumab alone. Interestingly, there was a rebound effect on vascular remodelling, with an augmentation in vessel size in all treatment groups compared to control.

Our results are comparable to the observations of a similar recent study where the authors demonstrated a significant reduction in vessel density and a simultaneous increase in vessel size\textsuperscript{324}. Furthermore, our histological analyses correlated well the advanced molecular imaging approaches as discussed in later chapters. Although no survival benefit was observed in favour of the novel
dual treatment strategy versus bevacizumab, we feel that if the survival study had being allowed to continue, a survival benefit would have being demonstrated. However, validation of the presented methods and results warrants further prolonged studies.
Chapter V

Bioluminescence & Pre-Clinical MRI:
In vivo imaging of anti-angiogenic inhibition in combination with a dual PI3K/mTOR inhibitor
5.1. Introduction

Neuro-imaging plays a significant role in the diagnosis of intra-cranial tumours and must consist of an assessment of location and extent of the tumour and of its biologic activity. Therefore, morphologic imaging modalities and functional, metabolic, or molecular imaging modalities should be combined for primary diagnosis and for following the course and evaluating therapeutic effects. MRI is the gold standard for providing detailed morphologic information and can supply additional insights into metabolism (MRS) and the microstructural environment (DWI) but still has limitations in identifying tumour grade, invasive growth into neighboring tissue, and treatment-induced changes, as well as recurrences.

With scientific progress, MRI gained importance in clinical, as well as in preclinical research. Firstly, MRI offers longitudinal in vivo studies without the need to sacrifice animals, thus making data better comparable and reducing the number of required animals. Secondly, in vivo MRI provides the possibility to monitor certain physiological parameters normally not assessable without invasive measures. Similarly, BLI is a well-established method for determining brain tumour growth and response to therapy in a quantitative fashion in orthotopic glioblastoma cell line–based rodent xenografts. Critically, the main advantages associated with BLI include scanner ease of operation, ability to evaluate several animals in a single image acquisition, minimal processing requirements, and relatively low equipment and running costs when compared to other imaging modalities, specifically MRI, PET and CT. Despite the ease of use, important limitations must be recognized when interpreting BLI data, primarily single two-dimensional image of the entire animal, which can present challenges in precisely localizing sites of BLI as well as poor spatial resolution.

Dynamic contrast-enhanced MRI (DCE-MRI) is in clinical use for the assessment of brain tumour vasculature. DCE-MRI measures changes in T1, T2 or T2* relaxivity due to leakage of a contrast agent into the extravascular space. Adapted MR sequences allow the calculation of parameters such as
blood or plasma volume, fraction of the extravascular/extracellular space, blood-to-tissue transfer constant or blood flow\textsuperscript{326,327}. Another approach was introduced by Dennie et al. based on the ratio of gradient and spin echo relaxation rate changes (ΔR*2/ΔR2) after injection of an iron oxide-based superparamagnetic contrast agent of high molecular weight\textsuperscript{328}. Since ΔR2*/ΔR2 increases with increasing vessel size, this method enables calculation of the average vessel size within a voxel, which reveals an additional important parameter of the angiogenic process. Based on these findings, Jensen and Chandra proposed to map the ratio of \( Q = ΔR2/(ΔR^*2) \)\textsuperscript{329}. Indeed, for a certain threshold of the concentration of the contrast agent, in combination with appropriate echo times, \( Q \) has the advantage of being independent of the concentration of the contrast agent\textsuperscript{329}. \( Q \) depends only on intrinsic tissue properties such as water diffusivity and has been shown to correlate with vessel density\textsuperscript{330,331}. Ullrich et al. improved the imaging protocols for vessel density and size calculation by including diffusion-weighted MR sequences to take into account that diffusion is not homogeneous inside tumour tissue\textsuperscript{332}.

Despite several modalities for in vivo imaging of small rodents exist, MRI remains widely advocated as the superior imaging modality in pre-clinical research. However, several preclinical studies have demonstrated a favourable correlation between bioluminescence and MR–based tumour assessment of drug response rates to novel treatments\textsuperscript{333,334}. In 2000, Rehemtulla et al demonstrated a similar reduction in tumour burden (BLI versus MRI) in a rat xenograft model treated with BCNU chemotherapy\textsuperscript{333}. More recently, BLI was successfully used to quantify the glioblastoma response to vector delivered VEGF receptors\textsuperscript{334}.
5.2. Chapter Aims

The aims of this chapter is to perform quantitative and qualitative analyses of tumour response to a novel dual PI3K/mTOR inhibitor / anti-angiogenic combination approach, using bioluminescence and advanced pre-clinical MR imaging.
5.3. Results

5.3.1. Bioluminescence

<table>
<thead>
<tr>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BEZ235</td>
<td>BEV</td>
<td>Combined Tx</td>
</tr>
</tbody>
</table>

Day 14, Day 28, Day 35, Day 42

BEZ235

BEV

Combined Tx
Figure 5.1a: Growth of intracerebral U87MG-Luc2 assessed using Bioluminescence. Images show tumour growth in a representative animal/group. No control mice survived to the end of the study.

B)

Figure 5.1b: Logarithmic BLI signal for each individual mouse/group.
Figure 5.1c: Logarithmic mean BLI signal ± SEM.

As demonstrated in Figure 5.1c, there was no difference between groups at day 35. A significant reduction in bioluminescence signal was observed at day 42 in the bevacizumab/Combination group in comparison to BEZ235 monotherapy. (p=0.040 ; p=0.040, respectively). No difference was demonstrated between bevacizumab and the combination group (p=0.548)
5.3.1 Volumetric Analysis

Pre - Treatment

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<td><img src="image5" alt="T2W CORONAL" /></td>
<td><img src="image6" alt="T2W CORONAL" /></td>
<td><img src="image7" alt="T2W CORONAL" /></td>
<td><img src="image8" alt="T2W CORONAL" /></td>
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Figure 5.2a: Pre-treatment growth assessment of intracerebral U87MG-Luc2 using T2-weighted MRI. Images show tumour growth in a representative animal/group. These representative mice are replicated throughout this chapter.
Figure 5.2b: Post-treatment growth assessment of intracerebral U87MG-Luc2 using T2-weighted MRI. Images show tumour growth in a representative animal/group.

Figure 5.3: Post-treatment median tumour volume ± range.
As demonstrated by Figure 5.3, all treatment strategies resulted in a reduction in tumour volume. Significant decreases in tumour volume (on T2w MRI images) were observed 3 weeks following treatment with BEV (94 % reduction, \( p = 0.0306 \)) and following treatment with the BEV/BEZ235 combination (97 % reduction; \( p = 0.0288 \)). [Note that more than one tumour was observed in some animals thus, where possible, tumours were analysed separately]. The reductions in tumour volume following treatment with the BEV/BEZ235 combination and following treatment with BEV alone were not significantly different. From the control group, 3 mice brains were imaged ex-vivo.

5.3.2. Cerebral Blood Volume

![Figure 5.4: Post-treatment Cerebral Blood Volume obtained using R2*MAP sequence. Images show CBV in a representative animal/group.](image)

Figure 5.4: Post-treatment Cerebral Blood Volume obtained using R2*MAP sequence. Images show CBV in a representative animal/group.
Figure 5.5: Post treatment ipsilateral median cerebral blood volume ± range.

As demonstrated by Figure 5.6, overall ipsilateral cerebral blood volume was reduced. Total tumour blood volume was significantly (47 %) lower in animals treated with BEV alone than in untreated animals (p = 0.0164). Total tumour blood volume was 51 % lower in animals treated with the BEV/BEZ235 combination than in animals treated with BEV alone (p = 0.0093).

5.3.3. Microvessel Cerebral Blood Volume (µCBV)

Figure 5.6: Post-treatment Microvessel Blood Volume obtained using R2 sequence. Images show µCBV in a representative animal/group.
As demonstrated in Figure 5.7, post treatment microvessel blood volume was reduced. Treatment BEV alone altered significantly the tumour microvessel blood volume compared to BEZ235 ($p = 0.0160$). Tumour microvessel blood volume was significantly (57%) lower in animals treated with the BEV/BEZ235 combination than in animals treated with BEV alone ($p = 0.0047$).
5.3.4. Vessel Size Index

![Vessel Size Index Images]

**Figure 5.8:** Post-treatment Vessel Size Index obtained using VSOP. Images show VSI in a representative animal/group.

![Vessel Size Index Graph]

**Figure 5.9:** Median Vessel Size Index ± range.
Vessel size was assessed using T2* mapping after SPIO contrast agent administration. No effect on tumour vessel size was observed in any treatment cohort.

5.3.5. Mean Density Index (MDI)

![Figure 5.10: Mean Density Index obtained using SPIO post contrast MRI. Images show MDI in a representative animal/group.](image-url)

Images show MDI in a representative animal/group.
As demonstrated by Figure 5.11, MDI was significantly (69 %) lower in animals treated with BEV alone and in the combination therapy compared to animals treated with BEZ235 alone (p = 0.0200 ; p = 0.0204). However, MDI was not reduced further in animals treated with the BEV/BEZ235 combination compared with animals treated with BEV alone.
5.3.6. Apparent Diffusion Coefficient (ADC)

Figure 5.12: Mean Apparent Diffusion Coefficient ± SEM.

As demonstrated by Figure 5.12, there was a non significant increase of ADC in the Bevacizumab/Combination treatment groups.
Figure 5.13: Correlation Coefficient MDI v vWF (Pearson’s correlation analysis).

As demonstrated in Figure 5.13, the mean density index determined via SSCE-MRI had a strong correlation with vWF.
5.4. Discussion

Intracranial pressure (ICP) results from a delicate relationship between CSF, blood and brain parenchyma. An invasive highly proliferative tumour, such as glioblastoma increases the brain parenchyma volume and interstitial fluid pressure, thereby reducing the CSF and/or cerebral blood flow in an attempt to offset the rising ICP. An elevated ICP is a neurosurgical emergency and is associated with a high morbidity and mortality. One of the several aims of surgical resection of a high grade tumour is to decompress the brain parenchyma and thus reduce the ICP. Consequently, an increasing tumour burden within the calvarium is a negative prognostic factor. In this chapter, we utilized a reproducible orthotopic explant model to assess tumour burden using bioluminescence and MRI.

As a baseline we preformed weekly bioluminescence over the study period to assess growth kinetics and tumour burden. Initially, pretreatment animals had comparable tumour sizes. As demonstrated previously, all of the vehicle control group died prior to the completion of the study. In addition, there was a clear reduction in the bioluminescence signal in the combination/bevacizumab alone group compared to BEZ235 monotherapy, on day 42.

Tumour growth was exponential in the non treated vehicle control group. Using T2w MRI, we have shown that treatment with BEV alone results in a pronounced decrease in tumour volume. A similar finding has been observed in patients, with effects evident within 2 weeks of treatment\textsuperscript{335}. However, our data suggest that the combination therapy does not further decrease GBM tumour volume compared to BEV monotherapy. Thus, it is possible that there is redundancy in the pro-growth signalling pathways inhibited by both BEV and BEZ235.
Over recent years, literature has well characterized the essential role of angiogenesis during tumour development, therefore treatments targeting vascular development will continue to be evaluated in preclinical and clinical studies. However, despite promising results in animal models, anti-angiogenic treatments in patients with gliomas have been largely disappointing, resulting in tumour resistance and the development of an infiltrative tumour phenotype. Furthermore, modern medicines limitations to accurately assess treatment responses to anti-angiogenic therapies makes key clinical decisions, pertaining to surgery, progression/pseudoprogression and/or chemo-radiotherapy difficult.

In an attempt to mechanistically interrogate the vasculature/angiogenesis of glioblastoma, we employed advanced neuro-imaging techniques in a preclinical setting.

Implementing SSCE-MRI, we have shown that the BEV/BEZ235 combination significantly reduces tumour blood volume and tumour microvessel volume, respectively, in comparison to BEV alone, probably due to BEZ235 mediated inhibition of tumour vessel signalling pathways. Most brain tumours over-secrete VEGF, which on binding and signalling through its receptor (VEGFR2) results in angiogenesis, vasculogenesis and abnormal permeabilization of the tumour vasculature. This hyperpermeability allows fluid to leak from the intravascular space into the brain parenchyma, which causes vasogenic cerebral oedema and increased interstitial fluid pressure. Herein we, and others elsewhere, have shown that treatment with BEV (which inhibits VEGFA binding to its receptor) reduces tumour vessel permeability. It is possible that synergistic inhibition of VEGFA/VEGFR2 binding (BEV) alongside inhibition of the VEGFR2 downstream PI3K/mTOR pathway (BEZ235) may explain the enhanced reduction in tumour blood volume we observed following the combination treatment.

BEV has previously been shown to decrease tumour blood volume as measured using gadolinium-based DCE MRI in a rat model of glioblastoma.
Moreover, patients with high-grade glioma with low tumour blood volume following treatment with BEV demonstrate improved progression-free survival compared with patients with high tumour blood volume. We hypothesize that the effect of the BEV/BEZ235 combination could translate to improved quality of life for glioblastoma patients by supporting reduced peritumoral oedema.

Despite the reduction of CBV, VSI was increased in all treated mice. Data elucidating the effects of BEV/BEZ235 on tumour blood volume and tumour microvessel volume are further supported by the vessel density reduction observed in the combination group as measured by vWF staining. Further studies are warranted to fully unravel the utility of MDI as a novel antiangiogenic imaging biomarker. Interestingly, we illustrated the use of VSOP as a potential biomarker of angiogenesis due to the significant correlation between the mean density index and vWF (Figure 5.12). As expected there was also an increase in the apparent diffusion coefficient in the treatment groups (NS). It should be recognized, however, that in untreated mice the cerebral vasculature is represented by aberrant tortuous thrombosed vessels with a complete lack of a hierarchical vessel network, thereby making accurate quantifiable measurements difficult in this group.

In addition, our results confirm the observations of several authors. In 2011 Von Baumgarten et al, employing intra-vital 2 photon microscopy, showed that bevacizumab inhibited the formation of new tumour vessels, which resulted in a marked reduction of vascular turnover as well as an increase in vascular diameter in a glioma mouse model. Recently, Viel et al demonstrated, using a rat orthotopic explant model, an increase in vessel size and cerebral blood volume with a concomitant reduction in vessel density and micro-circulation.

These studies, in addition to our findings, suggest that the micro-circulation/neo-angiogenesis is a key regulator of tumour growth kinetics in comparison to vascular remodelling specifically intussusception. Although many questions continue to surround the true efficacy of VEGF inhibitors in clinical practice, our
study demonstrates the additional antagonistic effect on tumour blood volume and micro-circulation of bevacizumab in combination with a PI3K/mTOR inhibitor and importantly, these effects can be mechanistically interrogated by improved molecular imaging approaches.
Chapter VI

Pre-Clinical PET: Tumour proliferation and angiogenic response to Bevacizumab in combination with a PI3K/mTOR Inhibitor
6.1 Introduction

In order to overcome the limitations of conventional MRI, molecular imaging with PET can be used to derive information on tumour hypoxia, necrosis, proliferative activity, permeability and/or vasculature. Since its inception in the early 1970’s, this functional imaging technique has gained widespread use within the realm of oncology and more recently as part of the peri-operative assessment of high grade gliomas. Radiolabeled amino acids are of particular interest for brain tumour imaging because of their high uptake in biologically active tumor tissue but low uptake in normal brain tissue. The most common radiotracers elicited for high grade gliomas are ([18F]FET) and [11C] Methionine (MET) and [18F]FLT.

In 1998, Shields and colleagues introduced 18FLT PET imaging as a noninvasive tool for visualizing tumor cell proliferation. There is strong emerging data supporting the use of 18FLT PET in a preclinical/clinical setting. In 2012, Corroyer-Dulmont et al, demonstrated that FLT was a sensitive imaging biomarker and detects early responses to therapy in a U87/U251 rat orthotopic model. Furthermore, the proliferative volume as determined by FLT correlated with overall survival in high grade gliomas in a prospective study involving 22 patients, while emerging data supports the potential use of 18F-FLT to predict anti-angiogenic treatment outcome in a clinical and preclinical setting. It should be noted, however, that 18F- FLT does not cross an intact brain-blood-barrier, therefore has limited function in the assessment of low-grade tumours. Indeed, care should be taken when interpreting FLT data, in isolation, in the presence of a benign lesion with BBB disruption as these cannot be distinguished from high grade lesions.

Currently, the radiopharmaceutical 18FET-PET is one of the most promising tracers and forms part of the multi-disciplinary approach for the management
of glioblastoma in many countries. In gliomas, $^{18}$F-FET uptake significantly correlates with tumor cell density and with microvascular density, permeability and neo-angiogenesis, all biological hallmarks of highly malignant glial tumours and has a half life of 110min.

Several studies have clearly indicated that $^{18}$F-FET PET in combination with MRI is able to improve the diagnostic and therapeutic assessment of patients with gliomas for neurosurgery\textsuperscript{258,259} and radiotherapy planning\textsuperscript{260}. Furthermore, PET can be a valuable parameter to assess treatment response and predict survival in the course of radiotherapy, chemotherapy and anti-angiogenic treatment as an adjunct to standard MRI. Stockhammer et al recently showed that $^{18}$F-FET PET seemed to be predictive for treatment failure in patients receiving bevacizumab earlier than MRI\textsuperscript{361}, while a retrospective analysis demonstrated that following the administration of TMZ concomitant with and adjuvant to RT in 79 patients with glioblastoma, the relapse pattern determined by $[^{18}$F$]$FET-PET was associated with MGMT methylation status, conveying a higher progression-free survival\textsuperscript{362}.

In this chapter we implemented advanced clinically relevant radio-tracers (FET,FLT) to interrogate the metabolic and angiogenic response following treatment of a dual PI3K/mTOR inhibitor in combination with VEGF inhibition, in an orthotopic glioma model.
6.2. Chapter Aims

The aim of this chapter is to investigate the changes in tumour metabolism/neo-angiogenesis in response to a novel dual PI3K/mTOR inhibitor/anti-angiogenic combination approach, using novel radiotracers as potential biomarkers of response.
6.3. Results

As demonstrated in Figure 6.1, there was a marked increase of $^{18}\text{F}$-FET uptake at week 6, compared to week 3 ($N=1$). No $^{18}\text{F}$-FLT imaging was performed in this group of mice at pre treatment due to malfunction of the cyclotron, or in the post treatment imaging session as all of the control mice had deceased. In addition, T2w MRI is also illustrated demonstrating the single mouse of this group who survived to $^{18}\text{F}$-FET imaging.

Figure 6.1: Control group - pre & post treatment PET imaging.
Figure 6.2: BEZ235 - pre & post treatment PET imaging. Images show PET imaging in a representative animal/group.
**Figure 6.3: Bevacizumab - pre & post treatment PET imaging.** Images show PET imaging in a representative animal/group.
Figure 6.4: Combined Tx - pre & post treatment PET imaging. Images show PET imaging in a representative animal/group.

As demonstrated in Figure 6.4, there was minimal FET and FLT uptake in the post treatment imaging session. The majority of the tracer uptake was located in the extra-dural space. No FLT imaging was carried out, at week 3, due to malfunction of the cyclotron.
6.3.1 Pre-Treatment $^{18}$F- FET

![Graph showing $^{18}$F-FET uptake across different groups: Control (N=4), BEZ 235 (N=5), Bev (N=3), Combined Tx (N=4).]

Figure 6.5: Pre - Treatment Mean $^{18}$F- FET ± SEM.

As demonstrated in Figure 6.5, $^{18}$F- FET uptake was comparable in all groups in the pre-treatment imaging session.

6.3.2 Pre - Treatment $^{18}$F- FLT

![Graph showing $^{18}$F-FLT uptake for BEZ 235 (N=4) and Bev (N=3).]

Figure 6.6: Pre - Treatment Mean $^{18}$F- FLT ± SEM.
As demonstrated in Figure 6.6, $^{18}$F- FLT uptake was comparable in BEZ235 and bevacizumab groups in the pre-treatment imaging session. As previously discussed, due to mechanical reasons, control and combined mice did not receive pre-treatment PET imaging.

6.3.3. Post-Treatment $^{18}$F- FET

![Graph showing $[^{18}F]$FET uptake comparison between groups.]

Figure 6.7: Post-Treatment Median $^{18}$F- FET ± Range.

$[^{18}F]$FET uptake was 46 % lower in animals treated with BEV alone (not significant) and 49 % lower in animals treated with the BEV/BEZ235 combination ($p = 0.0006$) than in animals treated with BEZ235 alone. $[^{18}F]$FET uptake was not significantly different between animals treated with BEV alone and those treated with the BEV/BEZ235 combination.
6.3.4. Post-Treatment $^{18}$F- FLT

Figure 6.8: Post-Treatment Median $^{18}$F- FLT ± Range.

No significant difference in $[^{18}F]$FLT uptake was observed between animals treated with BEV alone and those treated with the BEV/BEZ235 combination, supporting the MRI data. [Note that untreated animals did not survive until the post-treatment PET imaging session. $[^{18}F]$FLT uptake was significantly lower in animals treated with BEV alone and the BEV/BEZ235 combination compared to the animals treated with BEZ235 alone. ($p = 0.0021 \& 0.0118$, respectively)
As demonstrated in Figure 6.9, Ki-67 indexes were in good agreement with the T/B ratios of $^{18}$F-FLT uptake.

Figure 6.10: Correlation Coefficient $^{18}$F- FET v MDI (Pearson's correlation analysis).
As demonstrated in Figure 5.10, MDI showed strong correlation with the T/B ratios of $^{18}$F-FET uptake.

![Graph showing correlation between $^{18}$F-FET uptake and VSI](image)

Figure 6.11: Correlation Coefficient $^{18}$F- FET v VSI (Pearson’s correlation analysis).

As demonstrated in Figure 5.11, VSI did not correlate with the T/B ratios of $^{18}$F-FET uptake.
6.4. Discussion

Due in large part to their specificity and sensitivity, radiolabeled tracers are becoming advantageous in the peri-operative management of high grade gliomas. In order to investigate the proliferative and neovascular properties of glioblastoma in an orthotopic model, we implemented advanced clinically relevant radio-tracers (FET, FLT), in the pre and post treatment setting, in order to interrogate the metabolic and angiogenic response following treatment of a dual PI3K/mTOR inhibitor in combination with VEGF inhibition.

[18F]FET uptake is driven by large neutral amino acid transport and has been shown to correlate with L-type amino acid transporter expression (predominantly in the tumour cytoplasm and on the vascular endothelium), microvessel density and vessel formation in glioma. [18F]FET uptake has further been evaluated clinically as a prognostic marker of the response to BEV + irinotecan and has been shown to perform better than the RANO criteria in predicting treatment failure.

Pre - treatment [18F]- FET imaging demonstrated similar mean tumour uptake in all groups (Figure 6.5). Due to mechanical faults of the cyclotron, pre - treatment [18F]- FLT analysis was not possible in the control and combined treatment groups. However [18F]- FLT uptake was comparable in the BEZ235 and bevacizumab groups in the pre - treatment imaging session (Figure 6.6).

As previously discussed, no control mice survived to the end of the study, therefore no post treatment PET images were obtained for this group (Figure 6.7 & Figure 6.8).

Our study demonstrated a significant reduction in [18F]- FET uptake in the combined treatment group versus BEZ235 alone (p = 0.0204). Bevacizumab alone also reduced [18F]- FET uptake compared to BEZ235 alone, however this
was not significant (p = 0.0515). This indicates that bevacizumab alone and the combination group had a greater antagonistic effect on the vasculature and blood brain barrier (permeability) than BEZ235 alone. Moreover, there was a further reduction in $^{18}\text{F}$-FET uptake in the combined group versus bevacizumab alone, however this was non significant (p = 0.4881). Nevertheless, as $[18\text{F}]$FET also accumulates in actively metabolizing tumour cells, this finding would further support our earlier observations which suggest that the combination regimen does not enhance the direct tumour response.

Similarly, our study demonstrated a significant reduction in $^{18}\text{F}$-FLT uptake in the bevacizumab/combined treated groups compared to BEZ235 monotherapy (p = 0.0021 & 0.0118, respectively). In addition, $^{18}\text{F}$-FLT uptake was reduced in the combination group versus bevacizumab alone, however this was non significant (p=0.8689).

It is important to acknowledge the association of $[18\text{F}]$-FLT uptake in tumour tissue is dependent on BBB permeability. Bevacizumab, leads to “normalization” of tumour vessels and restoration of the BBB permeability. Therefore assessing anti tumour efficacity of anti-angiogenic treatment in glioblastoma, solely based on $[18\text{F}]$-FLT should be interpreted with caution.

Our results are in agreement with other studies that demonstrated a strong correlation between $^{18}\text{F}$-FLT and Ki67, as a marker of proliferation (Figure 6.9). Furthermore, $^{18}\text{F}$-FET uptake correlated well with MDI (SSCE-MRI), highlighting the ability of both $^{18}\text{F}$-FET and the novel contrast agent VSOP, to assess the microvasculature in response to anti-angiogenic therapy. While $^{11}\text{C}$-MET has being reported to correlate well with VSI$^{324}$, we demonstrated that $^{18}\text{F}$-FET did not reach significance with VSI (Figure 6.11).

This chapter demonstrates the ability of novel radiotracers to accurately assess physiological and biochemical information relating to angiogenesis and proliferation of glioblastoma in response to anti-angiogenic treatment. In addition, the combination of $^{18}\text{F}$-FET and $^{18}\text{F}$-FLT with SSCE-MRI provide an
accurate adjunct for monitoring treatment responses as well as improving our molecular understanding of glioblastoma.
Chapter VII

Discussion & Future Work
7.1. Glioblastoma

Glioblastoma is the most common primary adult brain tumour and is associated with a dismal prognosis. It is a diffusely infiltrating, aggressive brain tumour that has evaded past/current treatment strategies, and remains one of the most difficult oncological diseases to treat in the 21st century.

Approximately 200-220 patients are diagnosed with glioblastoma in the Republic of Ireland per year. In 1980, the median survival was 8 months. Presently, the median survival is 14.6 months with a five-year survival rate of only 5.1%, in spite of significant advances in our understanding and treatment of the disease.

The current standard of care for glioblastoma patients is maximum safe surgical resection combined with radiation and concomitant/adjuvant temozolomide (TMZ) therapy, commonly known as the Stupp protocol.

7.1.1. Surgery

The main objectives of surgery are to obtain a tissue diagnosis and to achieve the maximum safe surgical resection. The EOR plays a major role as an independent prognostic factor associated with improved overall and progression-free survival. To reach this objective, different technologies and surgical techniques have been introduced in neuro-oncological surgery, including neuronavigation systems, ultrasound, intraoperative MRI as well as intraoperative cortical and subcortical mapping techniques.

One novel compound used to guide surgical resection is 5-Aminolevulonic acid (5-ALA), which fluoresces and is preferentially taken up by tumour cells. This is administered orally two to three hours prior to surgery and reaches maximum florescence at 4 hours. Resection is performed using ultra-violet light to visualize the tumour, allowing for improved EOR.
7.1.2. Histopathology

Traditionally, glioblastoma have been classified into primary glioblastoma, believed to arise de novo, and secondary glioblastoma, believed to originate from the progression of a lower grade tumour. Each have individual molecular subtypes.

Primary glioblastoma are associated with amplification of the epidermal growth factor receptor (EGFR) porto-oncogene. They have also been associated with mutations in the promotor for telomerase reverse transcriptase (TERT) and the phosphate tensin homolog (PTEN) tumour suppressor gene. Secondary GBMs are associated with mutations in isocitrate dehydrogenase (IDH 1 and 2), the TP53 oncogene, and Alpha Thalassemia Mental Retardation Syndrome X-Linked (ATRX). Mutations in IDH 1 and 2 have been found in a large proportion of grade III tumours and are associated with a better prognosis\(^{93}\).

Genomic mapping by the Cancer Genome Atlas (TCGA) research network has identified four distinct molecular subtypes of GBM: classical, mesenchymal, proneural, and neural\(^{236}\). The classical subtype is associated an astrocytic expression pattern with EGFR amplification, chromosome 7 amplification and chromosome 10 loss, focal deletions of 9p, and TP53 mutations are not present. The mesenchymal subtype shows frequent mutations of NF1 and PTEN. The proneural subtype shows an oligodendrocytic expression pattern with amplification of chromosome 4q12, especially PDGFRA, and has the highest rate of IDH1 mutations. Lastly, the neural subtype contains alterations to EGFR amplification and PTEN deletion\(^{339}\). Further analysis of the molecular subtypes of glioblastoma is underway in order to better target specific treatment strategies against individual tumours, and future revisions to the WHO classification of brain tumours will likely reflect this.

7.1.3. Radiation
Following surgical resection, fractionated external beam radiotherapy plays an important role in the adjuvant treatment of GBM. A dose of 60 Gy of radiation is delivered in 30 fractions of 2 Gy to the involved area as well as up to 3 cm of the margin to treat infiltrating tumour. In older patients with a poorer functional status, the dose can be administered in fewer fractions.

The first major milestones in glioblastoma treatment came from radiotherapy. In 1976, the brain tumour study group (BTSG) 66-01 showed that whole brain radiotherapy extended mean survival by 5 months. A meta-analysis of BTSG 66-01, 69-01, and BTSG 72-01 showed poor survival at doses less than 45 Gy (3.1-4.2 months), improved survival at 50, 55, and 60 Gy (6.5, 8.4, and 9.8 months respectively). At doses greater than 60 Gy, there was neurotoxicity without any significant benefit. This forms the basis of the current dosing regime.

7.1.4. Chemotherapy

The addition of Temzolomide, a DNA alkylating agent, to the treatment protocol was an important milestone in treating GBM. In 2005, Stupp et al. showed that the adjuvant administration of concomitant Temzolomide and radiotherapy, followed by six cycles of Temzolomide, conferred a 2.5 month survival benefit over adjuvant radiotherapy alone. In addition, GBMs which were found to have methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) promotor sequence has been shown to provide a survival benefit in patients treated with adjuvant radiation therapy and Temozolamide.

The blood brain barrier (BBB) poses a significant challenge to delivering chemotherapeutic agents. It consists of endothelial cells, astrocytes, and pericytes, which regulate the transport of substances from the systemic to the cerebral circulation. Molecules are limited on their ability to cross the BBB based on size, polarity, and lipid solubility. In addition, transport proteins on the BBB promote efflux and restrict access. As a result of limited CNS penetration,
a number of systemic chemotherapeutic agents which are effective in other cancer types are not effective in treating glioblastoma\textsuperscript{341}.

A variety of approaches are being tested to improve chemotherapeutic drug delivery to the CNS. Osmotic disruption uses substances such as mannitol, infused into the carotid and vertebral arteries, to disrupt the tight junctions in the BBB to improve permeability\textsuperscript{342,343}. An alternative approach to disrupting the tight junctions of the BBB through the use of micro bubbles and transcranial low frequency focused ultrasound. In pre-clinical animal models, this has been shown to enhance drug delivery of Carmustine (BCNU)\textsuperscript{343}.

Drugs can bypass the BBB if they are directly administered into the CNS intrathecally, or through the use of polymer wafers. BCNU (Gliadel) wafers can be placed in the resection cavity during surgery to allow the slow release of BCNU into the CNS\textsuperscript{344}. However, there has been little evidence to support their use within the standard treatment regime.

\textbf{7.1.5. Targeted Therapy}

One strategy that has been considered is to inhibit tumour angiogenesis by means of targeting vascular endothelial growth factor (VEGF)\textsuperscript{177}. Historically, researchers tried to inhibit VEGF to prevent the tumour from recruiting a larger vascular supply and deny it resources required to grow. We now know that inhibiting VEGF normalizes the vasculature, which may improve the delivery of other chemotherapeutic agents\textsuperscript{345}. However, at the molecular level, the increase in tumour hypoxia causes the tumour to switch towards utilizing anaerobic glycolysis for energy. It also activates hypoxia-inducible factor 1α (HIF-1α) and other metabolic pathways resulting in enhanced tumour cell invasion\textsuperscript{230}.

A number of clinical trials have been performed using Bevacizumab, a monoclonal antibody against VEGF. Initial studies on patients with recurrent glioblastoma showed increased progression free survival at 6 months\textsuperscript{171,172}. Further investigation was performed to assess the efficacy of the antibody on
the initial treatment of glioblastoma, through the phase III AVAglio and the RTOG 0825 randomized control trials\textsuperscript{346,347}. These studies showed no benefit to adding Bevacizumab to the standard treatment protocol, although there was an increase in progression free-survival, as demonstrated in the AVAglio study only. In addition, the combination of Lomustine and Bevacizumab did not show any benefit in randomized phase III trials (EORTC 26101). This may be the result of the development of resistance to the treatment, and further studies are underway to identify the development of angiogenic escape mechanisms in glioblastoma.

Several other pathways are also dysregulated in GBM, including EGFR, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-beta), the PI3/AKT/mTOR pathway, and the RAS/MAPK pathway\textsuperscript{348}. Research is ongoing to target specific signalling receptors with monoclonal antibodies.

Vaccines have also been made against tumour specific antigens in order to produce a targeted immune response against the tumour cells. One such vaccine is Rindopepimut, which uses EGFRvIII as a target. Phase II trials demonstrated treatment efficacy, although it was unsuccessful in improving overall in a recent phase III randomized control trial (ACT IV).

7.1.6. Response to Treatment

It is important to be able to determine individual patients response to therapy in order to identify non-responders early in treatment. This enables clinicians to switch to an alternate strategy, as well as limits exposure to unnecessary side-effects.

Conventional imaging techniques remain the mainstay of assessing response to treatment as well as disease progression. Unfortunately, it is often difficult to differentiate between changes that result from a treatment and those that result from disease progression. This phenomenon, known as pseudoprog...
was first described 1979\textsuperscript{349}. It is thought to be the result of an inflammatory response to treatment secondary to vascular and glial injury\textsuperscript{350}. Radiologically, the features consist of T1 gadolinium enhancement and peritumoral oedema, which reflected the permeability of the the blood brain barrier.

An updated set of guidelines were developed by the Response Assessment in Neuro-Oncology (RANO) working group in 2010\textsuperscript{255}. This criteria uses T2, FLAIR, and post-contrast T1 sequences in order to assess whether imaging changes represent a treatment response, a stable change, or disease progression. In addition, MR diffusion and perfusion studies can help further differentiate between pseudoprogression and true progression by assessing neovascularization and microvascular leaks\textsuperscript{351}.

PET is increasingly used to supplement conventional neuroimaging in assessing disease progression. The tracer [\textsuperscript{18}F]FLT (3'-deoxy-3'-[\textsuperscript{18}F]fluorothymidine) was first used in 1998 by Shields et al.\textsuperscript{352} It is used to study cell proliferation in vivo, as it measures the uptake of thymidine into the pyrimidine salvage pathway. In addition, [\textsuperscript{18}F]FET PET tracers have been shown to correlate well with both 5-ALA uptake and contrast enhancement on MRI, especially for tumours with an intact BBB.\textsuperscript{353}
7.2. Hypothesis

We hypothesized that the combination of a novel dual PI3K/mTOR inhibitor and bevacizumab would convey potent anti-tumour effects in comparison to monotherapy strategies.

Therefore the aims of this study were two-fold:

- preform quantitative and qualitative analysis of tumour response to a novel dual PI3K/mTOR inhibitor & anti-angiogenic combination approach in a glioma model.

- Comparative analysis of advanced neuro-imaging modalities (pre-clinical MRI, PET), in a reproducible orthotopic model.
7.3 Discussion

Aberrant cell signalling networks commonly occur in glioblastoma tumours including the PI3K/AKT, giving rise to a pro-invasive and pro-angiogenic state. Despite our growing knowledge of these pathways, the molecular heterogeneity and reliance on multiple signalling pathways may explain why trials of single-agent molecularly targeted therapies have largely failed to demonstrate a survival benefit in patient populations. In this study, we implemented a mechanistic hypothesis-driven multimodality imaging approach to study the effects of dual targeting VEGF and PI3K/mTOR in an orthotopic model.

In this study we sought to measure tumour vessel size and density using dedicated sequences recently implemented by our group and others. Our approach facilitates the assessment of vessel parameters, total and microvessel tumour blood volume, VSI and MDI from $\Delta R2^*$, $\Delta R2$ and ADC maps. These MR sequences may be adapted for clinical translation. We believe that providing anatomical information on tumour vasculature could complement the physiological information provided by DCE MRI towards a more complete characterization of the tumour angiogenesis process. Unfortunately, due to practical (ie time) limitations during our experimental preclinical imaging procedures it was not feasible to include a DCE MRI protocol. Furthermore, the effect of gadolinium injections on VSOP T2/T2* relaxation is as yet unknown, as is any effect of VSOP on putative DCE MRI T1 relaxation.

Using T2w MRI, we have shown that treatment with bevacizumab alone results in a pronounced decrease in tumour volume. However, our data suggest that the BEV/BEZ235 combination does not further decrease glioblastoma tumour volume compared to BEV monotherapy. Thus, it is possible that there is redundancy in the pro-growth signalling pathways inhibited by both BEV and BEZ235. These observations are supported by Ki67 proliferation index and [18F]FLT uptake studies.
Using $\Delta R^2*$ and $\Delta R^2$ values, we have shown that the BEV/BEZ235 combination significantly reduces glioblastoma tumour blood volume and tumour microvessel volume, respectively, in comparison to BEV alone, probably due to BEZ235 mediated inhibition of tumour vessel signalling pathways. This hyperpermeability allows fluid to leak from the intravascular space into the brain parenchyma, which causes vasogenic cerebral oedema and increased interstitial fluid pressure. Herein we have shown that treatment with bevacizumab (which inhibits VEGFA binding to its receptor) reduces GBM tumour vessel permeability. It is possible that synergistic inhibition of VEGFA/VEGFR2 binding (BEV) alongside inhibition of the VEGFR2 downstream PI3K/mTOR pathway (BEZ235) may explain the enhanced reduction in tumour blood volume we observed following the combination treatment.

Data elucidating the effects of BEV/BEZ235 on tumour blood volume and tumour microvessel volume are further supported by the vessel density reduction observed in the combination group as measured by vWF staining. A corresponding trend towards decreased MDI was also observed in the combination group. Further studies are warranted to fully unravel the utility of MDI as a novel antiangiogenic imaging biomarker.

We also assessed tumour uptake of the novel tracer $[18F]$FET for monitoring therapy response. $[18F]$FET uptake is driven by large neutral amino acid transport and has been shown to correlate with L-type amino acid transporter expression (predominantly in the tumour cytoplasm and on the vascular endothelium), microvessel density and vessel formation in glioma. $[18F]$FET uptake has further been evaluated clinically as a prognostic marker of the response to BEV + irinotecan and has been shown to perform better than the Response Assessment in Neuro-Oncology (RANO) criteria in predicting treatment failure$^{278}$. We observed decreased $[18F]$FET uptake following BEV treatment, but failed to observe further reduced $[18F]$FET uptake following treatment with the BEV/BEZ235 combination. Nevertheless, as $[18F]$FET also accumulates in actively metabolizing tumour cells, this finding would further
support our earlier observations which suggest that the combination regimen does not enhance the direct tumour response.

vWF immunohistochemistry data suggested that all the treatments increased mean tumour vessel size. These findings agree with those of recent studies which showed increased tumour vessel size following BEV treatment in an orthotopic rat spheroid model, suggesting vessel normalization, maturation and pruning of immature vessels following treatment. Surprisingly, VSI was not affected by any of the treatments. Immunohistochemistry-derived VSI values have previously been shown to correlate with VSI values derived using the imaging method described here. However, this study did not assess VSI correlations after treatment with antiangiogenic agents. Further refinement of VSI as a robust imaging biomarker is required to decrease parameter variability.

The extensive preclinical imaging protocol employed has provided important information on the drug mechanism of action and further provided clues to potential clinical response. It is possible that translation of a BEV/BEZ235 combination regimen to the clinic could further reduce peritumoral oedema obviating the requirement for steroid treatment and improving patient quality of life.

We acknowledge that the sample size in the study is small and therefore underpower the statistical analysis. The intense imaging protocol, therapeutic approaches and aggressive tumour growth kinetics resulted in a small cohort of animals.

Additional preclinical studies using larger animal cohorts and clinically relevant orthotopic glioblastoma patient-derived xenograft models are warranted to extend and validate these findings. Nevertheless, extensive preclinical studies of this kind are increasingly necessary to refine clinical trial design, to better define the clinical setting where experimental regimens are likely to succeed, and to avoid long and costly clinical studies which may ultimately fail due to lack of response or toxicities. Implementing mechanistic hypothesis-driven pre-
clinical molecular imaging biomarker studies facilitates robust investigation of drug response. These data may more accurately predict the true clinical potential of novel therapeutic approaches.

7.4. Future Directions

7.4.1. Combination therapies as potential novel therapeutic agents.

Future therapeutic strategies will likely rely on a combination therapeutic approach targeting multiple cancer hallmarks. Despite the Cancer Genome Atlas (TCGA) research network performing whole genome sequencing of glioblastoma tumours, basic understanding of glioblastoma molecular pathways and aberrant escape pathways must be examined in greater detail eg C-MET & bevacizumab. Conversely, the continuing failure of bevacizumab to improve overall survival in patients with glioblastoma could be attributed to the overestimation of the importance of VEGF inhibition and inconceivably one could argue that future therapeutic strategies may not include VEGF inhibitors.

7.4.2. Implementation of clinically relevant advanced neuro-imaging techniques in a preclinical setting.

Develop a better understanding of the intricate cross-talk between signaling pathways, resistance patterns, tumour angiogenesis and tumour invasion. Enhanced understanding of the complex biology underlying glioblastoma cancer hallmarks may refine pre-clinical models thereby improving the outcome of translational work. Ultimately, a multimodal imaging approach will be necessary to fully interrogate the changes in response to different treatment options.
7.4.3. Development of a National PDX Database.

Approximately 200-220 operations were preformed on patients with glioblastoma per year (primary & recurrence), in Beaumont Hospital, the National Centre for Neurosurgery in the Republic of Ireland. Biobanking glioblastoma tissue would help to significantly improve our molecular understanding in the pre-clinical setting and ultimately lead to the development of clinically relevant glioma animal models. This database will commence January 2017 and has received financial support from Brain Tumour Ireland.

7.4.4. Clinically relevant glioma animal models.

A major rate limiting step of in vivo translational work is the application of a clinically relevant glioma model. The introduction of human derived glioblastoma cells in to immuno-compromised rodents would move away from the classical glioblastoma cell line derived studies, some of which were first introduced in the early 1970's. In addition, the implementation of surgical resection models of glioma would also provide further insight in to the possible discrepancies between clinical and preclinical results.

These models would provide a greater understanding of tumour dynamics in response to novel treatment options in conjunction with advanced clinical neuro-imaging techniques.

7.5. Conclusion

Although our understanding of the molecular mechanisms is rapidly expanding and many therapeutic strategies have been devised to arrest progression in the short-term, we have had little overall success in improving overall survival due to the development of aberrant escape pathways. Overall, this work demonstrates the potent anti-tumour/angiogenic effects of the combined therapeutic strategy, results which can be accurately interrogated with novel clinically advanced neuro-imaging techniques.
References


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have been implicated in glioblastoma multiforme disease progression. Oncogene. 1998; 17(13): 1755-7.


chemotherapy for recurrent gliomas. The Polymer-Brain Tumor Treatment Group. Lancet. 1995;345(8956):1008 – 1012.


356. Schwarzenberg J, Czernin J, Cloughesy TF, Ellingson BM, Pope WB, Geist C, et al. 3'-deoxy-3'-18F-fluorothymidine PET and MRI for early


Appendix I Ethical Approval

Royal College of Surgeons in Ireland
The Research Ethics Committee
121 St. Stephen’s Green, Dublin 2, Ireland.
Tel: +353 1 4022373 Fax: +353 1 4022449 Email: recadmin@rcsi.ie

Dr. David Smith, Acting Chair
Ms. Stephanie O’Connor, Convenor

Dr Annette Byrne,
Royal College of Surgeons in Ireland,
Department of Physiology and Medical Physics,
3rd Floor York House,
York Street, Dublin 2.

17th May, 2012

<table>
<thead>
<tr>
<th>Ethics Reference No:</th>
<th>REC728</th>
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<tr>
<td>Project Title:</td>
<td>Anti-angiogenic Efficacy of Bevacizumab alone and in combination with a dual PI3K/mTOR inhibitor in a reproducible orthotopic explant, using a multi-modality molecular imaging approach.</td>
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<tr>
<td>Researchers Name:</td>
<td>Dr Annette Byrne</td>
</tr>
</tbody>
</table>
| Other Individuals Involved: | Professor Jochen Prehn, Mr Phillip O’Halloran, Dr Helko Duessmann, Dr Gang Chen, Ms Monika Jarzabek, RCSI Department of Physiology and Medical Physics.
                       | Mr David O’Brien, Consultant Neurosurgeon, Beaumont Hospital, Professor Arnold Hill, Professor of Surgery RCSI. |

Dear Dr Annette Byrne,

Thank you for your Research Ethics Committee (REC) application. We are pleased to advise that ethical approval has been granted by the committee for this study. This letter provides approval for data collection for the time requested in your application and for an additional 6 months. This is to allow for any unexpected delays in proceeding with data collection. Therefore this research ethics approval will expire on 17th November, 2013. Where data collection is necessary beyond this point, approval for an extension must be sought from the Research Ethics Committee.

This ethical approval is given on the understanding that:
- All personnel listed in the approved application have read, understand and are thoroughly familiar with all aspects of the study.
- Any person using an animal for scientific purposes is in possession of a valid license from the Department of Health and Children for the project and has had the necessary documented training to conduct the work.
- A maximum number of 70 animals (mice) will be used for this study.
- Any significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the REC, and an ethical amendment submitted where appropriate.
- Please submit a final report upon completion of your project.

We wish you all the best with your research.

Yours sincerely,

PP Ms. Stephanie O’Connor (Convenor)
Dr David Smith (Acting Chair)
LAST—Ireland
www.Last-Ireland.org
5th-6th September 2011

This is to certify that

Philip O’Halloran

A skills attainment record should be presented with this certificate

(Course Content on the back of certificate)

Signed

Peter F. Nowlan
Course organiser
A new imageable orthotropic brain tumour model

**USING MOUSE MONITORING SCORE SHEETS**

- Refer to the scoring schedule in the record sheet for the frequency of scoring
- For assistance with assessing clinical scores refer to the score sheet index
- You can strike a line through redundant observations on the record sheet if necessary
- When an animal reaches a total score of '4' from multiple observations or a score of '3' for a single observation, it is to be euthanased immediately
- Score sheets should be kept in the animal room during the study and archived after the study

<table>
<thead>
<tr>
<th>Observation</th>
<th>Score of 0</th>
<th>Score of 1</th>
<th>Score of 2</th>
<th>Score of 3</th>
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<tbody>
<tr>
<td>Skin</td>
<td>Normal</td>
<td>Slight discoloration / pale.</td>
<td>Minor lesions</td>
<td>Extensive skin lesion(s)</td>
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<tr>
<td>Activity</td>
<td>Normal</td>
<td>Isolated</td>
<td>Huddled/Inactive / abnormal posture (short-term effects observed post IP treat as flawed injection)</td>
<td>Moribund Huddled/Inactive</td>
</tr>
<tr>
<td>Breathing</td>
<td>Normal</td>
<td>Rapid, shallow</td>
<td>Rapid, shallow, abdominal</td>
<td>Laboured, gasping, abnormal breathing</td>
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<tr>
<td>Movement</td>
<td>Normal</td>
<td>Uncoordinated walking</td>
<td>Reluctant to move anytime; if provoked does not move. (short term effects observed post IP treat as flawed injection)</td>
<td>Staggering, paralysis, limb dragging</td>
</tr>
<tr>
<td>Condition/ Cachexia</td>
<td>Normal</td>
<td>Observed 72h post treatment Mild loss of muscle Vemtorose visible</td>
<td>Observed 96h post treatment Loss of fat, no growth, cachexia</td>
<td>Substantial cachexia over 1 week</td>
</tr>
<tr>
<td>Dehydration</td>
<td>Nil</td>
<td>Skin less elastic Mild loss of skin flexibility</td>
<td>Skin tents Moderate loss of skin elasticity</td>
<td>Eyes sunken severe loss of skin elasticity</td>
</tr>
<tr>
<td>Body Weight</td>
<td>Normal</td>
<td>&gt;5% weight loss</td>
<td>Weight loss &gt;15%</td>
<td>&gt;20% loss over 1 week observation period</td>
</tr>
<tr>
<td>Faeces/Urine</td>
<td>Normal</td>
<td>Loose faeces</td>
<td>Diarrhoea, blood stained urine</td>
<td>Consistent blood stains</td>
</tr>
</tbody>
</table>
Appendix II  Relevent Publications

Appendix III Oral Presentations


- The Molecular Muddle of Glioblastoma: understand the neovascular interactions Plenary Session. Sir Peter Freyer Surgical Meeting. September 2013.


**Irish Neurosciences Association Meeting.** Cork. May 2012.
Appendix IV  Poster Presentations


Appendix V  Prizes/Grants Awarded

- British Association of Cancer Research Student Travel Award. August 2013.
- Winner of the Kamal Sayed Neurosurgical Research Prize. RCSI. March 2013
- Finalist at St. Lukes Oncology Young Investigators Award. January 2013.
- European Bioimaging Group: Research Fellowship Grant. October 2012.
- Winner of Neurosurgery Prize at Irish Institute of Clinical Neurosciences. May 2012.