Characterisation of cytokine secretion in malignant gliomas

by

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Declaration

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work.

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Abbreviations

AA	Anaplastic astrocytomas
Akt	Protein kinase B
Ang	Angiopoietin
APC	Allophycocyanin
APC	Antigen presenting cell
BBB	Blood-brain barrier
BCL-2	B cell lymphoma 2
BCNU	β-chloro-nitrosourea
bFGF	Basic fibroblast growth factor
BTNW	Brain tumour north west
CCL-2	CC chemokine ligand 2
CCNU	Chloroethyl-cyclohexyl-nitrosourea
CD	cluster of differentiation
CDKN2A/ARF	Cyclin-dependent kinase inhibitor – Alternative reading frame
CGH	Comparative genomic hybridisation
CIK	Cytokine induced killer
CMI	Cell mediated immunity
CNS	Central nervous system

COX-2	Cyclo-oxygenase 2
CSF	Cerebro-spinal fluid
CSF-1	Colony stimulating factor 1
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
DAB	Diaminobenzidine
DMSO	Di-methyl sulphoxide
DNA	Deoxyribose nucleic acid
dsRNA	Double stranded ribo-nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGFRViii	Epidermal growth factor receptor variant 3
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
EPC	Endothelial progenitor cells
FADD	Family adapter
Fas	Fatty acid synthetase
FcR	Fragment crystallisable receptor

FGF- basic	Fibroblast growth factor basic
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
GBM	Glioblastoma multiforme
G-CSF	Granulocyte – colony stimulating factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage – colony stimulating factor
GTP	Guanine triphosphate
H&E	Haematoxylin and eosin
HB-EGF	Heparin binding epidermal growth factor
HDAC	Histone deacetylases
HGF	Hepatocyte growth factor
HGF/SF	Hepatocyte growth factor/scatter factor
HIF-1	Hypoxia inducible factor-1
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus 1
ICA	Internal carotid artery

ICAM-1	Intercellular adhesion molecule 1
ICD-O	International classification of diseases for oncology
IDH	Isocitrate dehydrogenase
IDO	Indolamine 2,3-dioxygenase
IGFBP-1	Insulin like growth factor binding protein 1
IL-x	Interleukin
INF-γ	Interferon gamma
IP-10	Interferon-inducible protein 10
IRAK	Interleukin 1 receptor-associated kinase
IRES	Internal ribosomal entry site
Jak-STAT	Janus kinase signal transducer and activator of transcription
Ki67/MIB-1	Kiel 67 protein
LAK	Lymphokine activated killer
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid derived suppressor cells
MGMT	O-6-methylguanine-DNA methyltransferase

MHC	Multi-histocompatibility complex
miR-21	Micro RNA 21
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRS	Magnetic resonance spectroscopy
MT1-MMP	Membrane type 1 matrix metalloprotease
mTOR	Mechanistic target of rapamycin
NF2	Neurofibromin 2
NF-kB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
ObR	Obesity receptor
OS	Overall survival
P14ARF	Protein 14 Alternative reading frame
P16	Protein 16
P19ARF	Protein 19 alternative reading frame
P21	Protein 21
p53	Protein 53
PA	Pilocytic astrocytomas

PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed death
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death ligand 1
PE	Phyco-erythrin
PECAM-1	Platelet endothelial cell adhesion molecule -1
PET	Positron emission tomography
PFS	Progression free survival
PGE2	Prostaglandin E2
PLGF	Placental growth factor
PMA	Pilomyxoid astrocytoma
pRB	Retinoblastoma protein
PRL-R	Prolactin receptor
PTEN	Phosphatase and tensin homolog
PXA	Pleomorphic xanthoastrocytoma
Ras	Rat sarcoma

RCF	Relative centrifugal force
RPMI	Roswell Park memorial institute
RT-PCR	Real time polymerase chain reaction
SCF	Stem cell factor
SEGA	Subependymal giant cell astrocytoma
sHER2-neu	Soluble human epidermal growth factor receptor – neural
sIL-6R alpha	Soluble interleukin 6 receptor alpha
SPECT	Single photon emission computed tomography
SSC	Side scatter
STAT 3	Signal transducer and activator of transcription
TAM	Tumour associated macrophages
TGF	Transforming growth factor
T_H	T Helper cell
TIE	Tumour immune escape
Tie-2	Tyrosine-protein kinase receptor 2
TIS	Tumour immune surveillance
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TP53	Tumour protein 53

TRAF	TNF receptor associated factor
Treg	Regulatory T cell
TSC	Tuberous sclerosis complex
TSP-1	Thrombospondins -1
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WBC	White blood cell count
WHO	World Health Organisation

Glossary of technical terms

Abluminal – outer surface of a blood vessel

Alkylating agent – therapeutic compound that adds an alkyl group to a replicating DNA strand thereby preventing cell reproduction

Angiogenesis – Formation of new blood vessels

Calcification – deposition of calcium in tissue

Comparative genomic hybridisation - cytogenetic method for assessing copy number mutations in disease

Cortical – The outer layer of the cerebrum

Epigenetic – genetic changes that do not involve a change in DNA sequence

Fluorescent in situ hybridisation – cytogenetic technique that utilises fluorescent probes to identify specific genes

Gemistocyte – an astrocyte swollen through inflammation

Haemopoiesis – formation of new blood cells

Hyperostosis – deposition of calcium leading to bone formation in ligaments

Kaplan-Meier curve – graphical representation of survival data in medical statistics

Loss of heterozygosity – chromosomal mutation resulting in the loss of a whole gene

Lumial – inner surface of a blood vessel

Methylation – deposition of methyl groups to a promotor region of a gene repressing its transcription

Microvascular hyperplasia – enlargement of new blood vessels

Mitoses – cells displaying evidence of mitotic cell division

Multivariate analysis – statistical technique used to analyse data that arises from more than one variable.

Myelosuppression – suppression of white blood cell production

Myxoid – containing mucin. Used to describe histological appearance of tissue

Necrosis – death of cells due to disease

Nuclear atypia – cells with unusually shaped nuclei

Papilledema – swelling of optic nerve due to increased inter-cranial pressure

Parenchyma – An organ's functional tissue

Perinuclear halo – vacuolated area around the nucleus formed when the nucleus shrinks

Pleomorphism – cells in a tissue of different shapes and sizes

Quiescent – cells in a dormant or non-reproducing state

Sagittal, coronal and axial dimensions – anatomical planes used in imaging

Sensorineural – hearing loss due to damage to the inner ear

Stereotactic – surgery utilising 3-dimensional imaging techniques to locate a point within the body eg. A tumour to be removed.

Supratentorial – Area of the brain above the tentorium cerebelli containing the cerebrum

Univariate analysis - statistical technique used to analyse data that arises from one variable.

Abstract

Cytokines are proteins produced by cells of the immune system. The working hypothesis of the thesis is to show that the lymphocytes and glial cells in Glioblastoma Multiformae patients have an altered pattern of secretion of cytokines compared with low-grade and non-cancerous patients. Lymphocyte subset analysis was performed using flow cytometry. Serum specimens taken from both high and low grade glioma patients were analysed with samples taken from control patients with no history of cancer. Cerebro-spinal fluid was analysed from high-grade glioma patients. Comparisons of high grade pre-surgery serum samples, high grade post-surgery samples, control pre-surgery samples and control post-surgery samples were included. The analysis was completed utilising a luminex immunoassay. This technology is able to measure 34 cancer-associated analytes simultaneously.

Immunohistochemistry of candidate biomarkers was done using primary tumour tissue. The results show that there were significant differences in several analytes in the sera and CSF of the different groups. These were follistatin, fibroblast growth factor (FGF), granulocyte – colony stimulating factor (G-CSF), soluble human epidermal growth factor receptor 2 neural (sHER2neu), soluble interleukin-6 receptor alpha (sIL-6R alpha), platelet-derived growth factor - AABB (PDGF-AABB), platelet and endothelial cell adhesion molecule (PECAM-1), stem cell factor (SCF), prolactin, soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) and urokinase plasminogen activator (uPA). The data also show that tumour tissue revealed increased expression of follistatin and G-CSF with little expression of

prolactin. In conclusion, these results suggest that potential candidate biomarkers can be used to enable diagnosis of glioma and moreso to distinguish between different grades of glioma using a panel of biomarkers.

Chapter 1

Introduction

1.1 Cancer

A cancer can be described by a group of diseases characterised by abnormal cell growth and a loss of control of proliferation. A tumour or neoplasm is usually an abnormal growth of cells that usually form a concentrated mass and can be either benign or malignant. A benign tumour does not spread (metastasise) to other parts of the body whereas a malignant tumour can proliferate through either local, lymphatic or haematogenous spread. A cancer can be defined as a malignant neoplasm with the potential to metastasise. All malignant tumour cells display the six hallmarks of cancer (Hanahan and Weinberg 2011). These include;

- Self-sufficiency in growth signals
- Insensitivity to anti-growth signals
- Evasion of apoptosis (cell death)
- Potential for limitless replication
- The ability for sustained angiogenesis
- Tissue invasion and metastasis

Some authors report a seventh hallmark of cancer, that of cancer-related inflammation in the tumour micro-environment (Bonomi, et al. 2014).

1.2 Glioma

A glioma is a tumour that originates in the central nervous system (CNS), specifically the glial cells. Glial cells comprise the non-neuronal cells of the brain and spinal cord and perform specific functions. These functions include the stabilisation of neurones, the metabolism of neuroactive molecules, the control and regulation of neurotransmission and the destruction of pathogens

and ‘dead’ neurones. Around 50% of brain tissue is made up of glial cells with roughly equal numbers of neuronal cells (Azevedo, et al. 2009). Glioma cells can be divided into 3 types, namely, astrocytes, oligodendrocytes and ependymal cells.

1.2.1 Epidemiology of glioma

A glioma is the most common form of brain tumour. Table 1.1 provided by Cancer Research UK shows the incidence of all brain tumours in the UK in 2013. The rates given are both the crude rate and the age-standardised rate to account for the different demographic constitution of the UK regions. This is done as increasing age is the most significant risk factor for getting a brain tumour (Ohgaki & Kleihues 2007) and enables a more accurate comparison between the regions.

Table 1.1 Number of new cases and rates of brain and other central nervous system (CNS) tumours, UK 2013 taken from by Cancer Research UK.

Brain, Other CNS and Intracranial Tumours (C70-C72, C75.1-C75.3, D32-D33, D35.2-D35.4, D42-D43, D44.3-D44.5): 2013

Number of New Cases, Crude and European Age-Standardised (AS) Incidence Rates per 100,000 Population, UK

		England	Wales	Scotland	Northern Ireland	UK
Male	Cases	4,312	311	464	77	5,164
	Crude Rate	16.3	20.5	17.9	8.6	16.4
	AS Rate	18.2	21.7	19.8	9.9	18.3
	AS Rate - 95% LCL	17.7	19.3	18.0	7.7	17.8
	AS Rate - 95% UCL	18.8	24.1	21.6	12.1	18.8
Female	Cases	4,487	387	519	67	5,460
	Crude Rate	16.4	24.7	18.9	7.2	16.8
	AS Rate	16.9	24.0	18.9	7.8	17.2
	AS Rate - 95% LCL	16.4	21.6	17.3	5.9	16.7
	AS Rate - 95% UCL	17.4	26.4	20.5	9.7	17.6
Persons	Cases	8,799	698	983	144	10,624
	Crude Rate	16.3	22.6	18.5	7.9	16.6
	AS Rate	17.5	23.0	19.3	8.9	17.7
	AS Rate - 95% LCL	17.2	21.3	18.1	7.4	17.4
	AS Rate - 95% UCL	17.9	24.7	20.5	10.3	18.0

95% LCL and 95% UCL are the 95% lower and upper confidence limits around the AS Rate

http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/brain-other-cns-and-intracranial-tumours/incidence#heading-Zero Website accessed 17/10/16

Within these tumour groups the glioma group makes up about half of all brain tumours (Table 1.2).

Table 1.2 Incidence of brain and other CNS tumours by morphology taken from Cancer Research UK.

Brain, Other CNS and Intracranial Tumours by Morphology: 2006-2010

Proportion of New Cases, England

Morphological Group	% of all Brain, other CNS and intracranial tumour cases	% of these more aggressive	% of these less aggressive
Astrocytomas	34%	95%	5%
Meningiomas	21%	8%	92%
Pituitary	8%	1-2%	98-99%
Gliomas unspecified	6%	*	*
Cranial and paraspinal nerve tumours	6%	5%	95%
Oligodendrogliomas	3%	*	*
Ependymomas	2%	75%	25%
Embryonal tumours	2%	100%	0%
Other tumour types	5%	*	*
Unknown or unspecified type	14%	*	*

* It is not possible to break down these heterogeneous groups by level of aggressiveness

http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/brain-other-cns-and-intracranial-tumours/incidence#heading-Four Website accessed 17/10/16.

Data in the literature show that the incidence of glioma is increasing constantly, (Arora et, al 2010). Gliomas have the capacity to infiltrate the CNS and are difficult to surgically extirpate. This is one of the reasons for the high levels of tumour recurrence following current treatments and the associated patient mortality (Prados and Levin 2000).

The World Health Organisation (WHO) produces a definitive classification and grading of tumours that is accepted worldwide. This is currently on its fifth

edition and was published in 2016 (Louis, et al. 2016). The classification criteria includes information on molecular parameters as well as histology, epidemiology, clinical signs and symptoms, imaging, prognosis and predictive factors and gives each classification an International classification of diseases for oncology (ICD-O) code, (table 1.3).

Table 1.3 WHO classification of tumours of the CNS taken from Louis et al. (2016).

WHO classification of tumours of the central nervous system

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M–mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*
Other astrocytic tumours	
Pilocytic astrocytoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tanycytic ependymoma	9391/3
Ependymoma, *RELA* fusion–positive	9396/3*
Anaplastic ependymoma	9392/3
Other gliomas	
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	9431/1
Astroblastoma	9430/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	9390/1
Choroid plexus carcinoma	9390/3
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease)	9493/0
Desmoplastic infantile astrocytoma and ganglioglioma	9412/1
Papillary glioneuronal tumour	9509/1
Rosette-forming glioneuronal tumour	9509/1
Diffuse leptomeningeal glioneuronal tumour	
Central neurocytoma	9506/1
Extraventricular neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma	8693/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	9395/3
Embryonal tumours	
Medulloblastomas, genetically defined	
Medulloblastoma, WNT-activated	9475/3*
Medulloblastoma, SHH-activated and *TP53*-mutant	9476/3*
Medulloblastoma, SHH-activated and *TP53*-wildtype	9471/3
Medulloblastoma, non-WNT/non-SHH	9477/3*
Medulloblastoma, group 3	
Medulloblastoma, group 4	
Medulloblastomas, histologically defined	
Medulloblastoma, classic	9470/3
Medulloblastoma, desmoplastic/nodular	9471/3
Medulloblastoma with extensive nodularity	9471/3
Medulloblastoma, large cell / anaplastic	9474/3
Medulloblastoma, NOS	9470/3
Embryonal tumour with multilayered rosettes, C19MC-altered	9478/3*
Embryonal tumour with multilayered rosettes, NOS	*9478/3*
Medulloepithelioma	9501/3
CNS neuroblastoma	9500/3
CNS ganglioneuroblastoma	9490/3
CNS embryonal tumour, NOS	9473/3
Atypical teratoid/rhabdoid tumour	9508/3
CNS embryonal tumour with rhabdoid features	*9508/3*
Tumours of the cranial and paraspinal nerves	
Schwannoma	9560/0
Cellular schwannoma	9560/0
Plexiform schwannoma	9560/0

As gliomas are primary brain tumours derived from three types of glial cells, namely astrocytes, oligodendrocytes and ependymal cells then for the purposes of this study the research focused on those gliomas from astrocytic, oligodendroglial, oligoastrocytic and ependymal origin.

1.3 Glioma types

1.3.1 Astrocytic tumours

Astrocytes are star-shaped glial cells that perform many supporting functions for the neuronal cells. Tumours from an astrocytic origin are the most common type of brain tumour with Glioblastoma comprising 34% of malignant tumours of the CNS (Cancer Research UK accessed September 2016).

Astrocytomas can be separated into two distinct groupings. The first group has a circumscribed narrow zone of infiltration which include, pilocytic astrocytoma, subependymal giant cell astrocytoma and pleomorphic xanthoastrocytoma. The second group has a more diffuse zone of infiltration and include low grade astrocytomas, anaplastic astrocytoma and glioblastoma. These groupings reveal differences in cellularity, cellular pleomorphism, the degree of neovascularisation and the presence of necrosis (Desbaillets,et al. 1997).

1.3.1. a. Diffuse astrocytoma

These astrocytomas are slow growing tumours that frequently transform into a more malignant phenotype and are classified as a WHO grade II tumour. They are almost always fatal with median survival data ranging from 2 to 10 years with incidence between the sexes being approximately equal. Patients

with these tumours are presented with changes in behaviour, language use and seizures. Upon histological examination these tumours show a well-differentiated, hypercellular population of astrocytes with minimal pleomorphism and no mitotic activity. There is no microvascular proliferation and necrosis. Tumour borders are usually poorly defined with frequent perineuronal satellitosis (Pignatti et al. 2002).

These tumours can be divided into three types namely: fibrillary, gemistocytic and protoplasmic depending on the histological appearance and each carry a different prognosis. Those tumours that have a significant gemistocytic component have a significantly poorer prognosis (Krouwer et al. 1991). Protoplasmic astrocytomas are histologically most frequently confused with fibrillary astrocytomas and they have a different incidence, being diagnosed in males at a younger age than in fibrillary astrocytoma (Prayson and Estes 1995).

1.3.1. b. Anaplastic astrocytoma

Anaplastic astrocytomas (AA) are defined as a malignant glioma and classified as a WHO grade III tumour. These are diffusely infiltrating lesions with focal or dispersed regions of anaplasia and marked proliferative potential (Arko et al. 2010) They typically display hypercellularity, cellular pleomorphism, nuclear atypia and microvascular proliferation. The median survival time ranges from 2–3 years. This is affected by the amount of time it takes for the tumour to progress to grade IV (Louis et al. 2007b). Anaplastic astrocytomas are most common between the ages of 40 to 50 years with a median age at diagnosis of 41. Anaplastic astrocytomas usually originate from

lower grade astrocytomas and transform into a malignant secondary glioblastoma through a series of mutations including the p53 mutation and a loss of heterozygosity (LOH) on chromosome 10q and 19q (Ichimura et al. 2004;Von Deimling et al. 1994). Figure 1.1 outlines the genetic pathways in the evolution of primary and secondary glioblastoma.

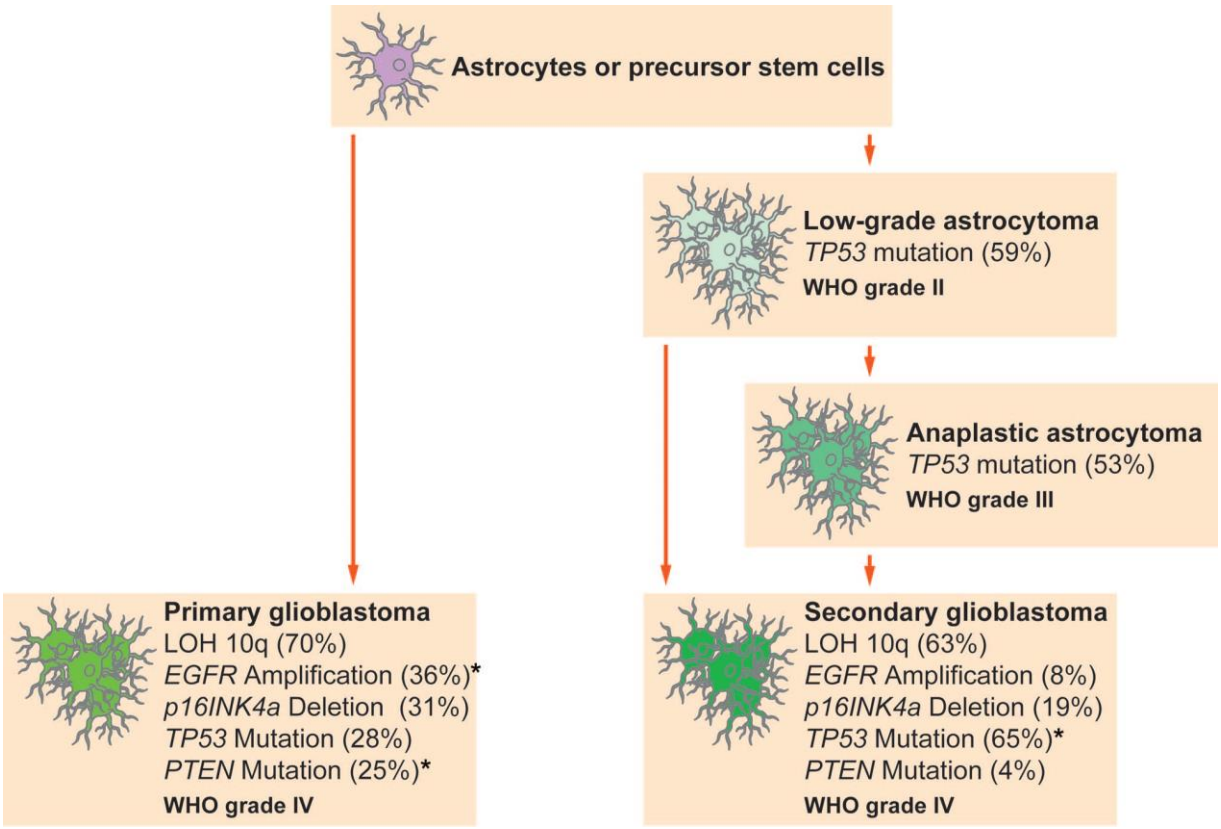


Figure 1.1 Flow diagram showing the genetic pathways operative in the evolution of primary and secondary glioblastoma. Taken from Ohgaki and Kleihues (2007).

1.3.1. c. Glioblastoma Multiforme (GBM)

Glioblastoma multiforme is the most prevalent and malignant of all primary brain tumours with an incidence of about 4-5 new cases per 100,000 population per year (Louis et al. 2016). The prognosis for such patients is poor with an average survival of 12 months from diagnosis (Ray-Chaudhury 2010). This disease can occur at any age although generally it is a disease of

adulthood and as they are highly infiltrative they are classified as a WHO grade IV tumour (Louis et al. 2016). There is a slight bias towards male predominance (Ray-Chaudhury 2010).These tumours tend to be supratentorial with a diffuse infiltration into the brain parenchyma.

The prognosis of these patients is poor. The most important prognostic factor is age with patients less than 50 years at diagnosis having a better prognosis (Louis et al. 2016). Other prognostic indicators include duration of symptoms, extent of resection and necrosis. (Ohgaki et al. 2006).

1.3.2 Glioblastoma variants

There are some cellular variants of GBMs that are also classified as WHO grade IV tumours. These are giant cell glioblastoma and gliosarcoma but these are not part of this study.

1.3.3 Oligodendroglial tumours

These tumours are well-differentiated, indolent neoplasms of the central nervous system that have a favourable prognosis. Median survival ranges from 6-10 years. They are encountered less frequently than astrocytomas, however data suggest that they are more common than previously thought with estimates ranging up to 25% of primary brain tumours (Coons et al. 1997). They can be separated into anaplastic and non-anaplastic forms.

1.3.4 Oligodendroglioma

These are WHO grade II tumours that originate from either oligodendrocytes or from glial precursor cells. They frequently demonstrate a moderate cellularity with rare mitotic activity. Tumour cells are diffusely infiltrating into

both grey and white matter with rounded nuclei giving a diagnostic ‘fried egg’ appearance. There is no necrosis or vascular proliferation, however, there is usually a network of calcified blood vessels that gives a ‘chicken wire’ appearance. If calcification is present then this can be a distinguishing feature of these tumours from astrocytomas. This can lead to spontaneous haemorrhage resulting in headache, confusion and lethargy as presenting features. The most frequent symptom of these tumours is seizures due to the frontal lobe involvement in 50% of cases (Jenkins et al. 2006).

Molecular analysis of these tumours has revealed a LOH on the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) which correlates with improved survival (Jenkins et al. 2006).

1.3.5 Anaplastic oligodendroglioma

An anaplastic oligodendroglioma corresponds to a WHO grade III malignancy and is rarer than its less malignant oligodendroglial counterpart accounting for around 3.5% of malignant gliomas (Louis et al. 2016). In addition to the histological features of oligodendroglioma, anaplastic oligodendrogliomas may demonstrate areas of tumour necrosis (Louis et al. 2016). Anaplastic oligodendrogliomas may demonstrate an increased cellularity, cytological atypia, and high mitotic activity that presents as microvascular proliferation.

Anaplastic oligodendrogliomas, in addition to the 1p and 19q chromosomal loss of the WHO grade II tumour also frequently demonstrate further deletions and genetic modifications including LOH at 10q, *EGFR* amplification, and *CDKN2A/ARF* deletion (Ferrer-Luna et al. 2011). There is a similar clinical presentation to oligodendroglioma as well as a preference for the frontal lobe.

1.3.6 Oligoastrocytic tumours

Oligoastrocytomas are tumours containing varying proportions of oligodendrocytes and astrocytes.The astrocytes usually correspond to diffuse astrocytoma cells of WHO grade II. Oligoastrocytic tumours are also classified as WHO grade II. Previously, diagnosis was made upon the presence of 25% of any single cell type (Smith et al. 1983), however today any mixture of each tumour type can be considered an oligoastrocytoma. These tumours can, like oligodendrogliomas and astrocytomas, be divided into low grade and high grade(anaplastic) variants, the latter characterized by increased cellularity, mitotic activity, microvascular proliferation and necrosis.

1.3.7 Oligoastrocytoma

These tumours are typically found in middle-aged patients with a small bias towards males. They are usually supratentorial and located in the frontal lobe presenting with seizures (Zhang et al. 2014).

Prognosis is estimated at 37.6 months for anaplastic gliomas, however, due to the heterogenous nature of these tumours this prognosis varies between less than 6 months and greater than 5 years (Zhang et al. 2014). A greater prognosis is associated with a younger age at operation, gross total tumour resection, and postoperative radiation therapy (Zhang et al. 2014). It has been found that the greater the oligodendroglial component in such a mixed tumour, the better prognosis and smoother clinical course since oligodendroglial tumours tend to be less invasive with a greater chance of resection. The IDH-1 mutation status included in the Louis 2016 classification is demonstrated through the mutant

gene being associated with grade I and grade II tumours and rarely in anaplastic forms. It is also associated with younger, secondary GBMs and better outcomes (Zhang 2014).

1.3.8 Anaplastic oligoastrocytoma

These tumours typically affect the same group of people as their low-grade counterparts with supratentorial lesions, also presenting with seizures. Histological features are also similar. Clinical outcome is inferior however with median survival time much shorter of 1.25 years. (Buckner et al. 2007).

1.3.9 Ependymal tumours

Ependymomas are tumours that derive from the ependymal lining of the ventricular system as well as from the central canal of the spinal cord. They are usually found in children and form 8-10% of paediatric CNS tumours. They are also present in adults where they form 1-3% of CNS tumours. In adults most (75%) of the tumours originate from the spinal canal.

Histopathological features include cells from both glial and epithelial sources with neoplastic cells surrounding blood vessels. These are the source of the diagnostic perivascular pseudorosettes in which tumor cells are arranged around vessels with an intervening zone consisting of thin ependymal processes directed toward the wall of the vessel (Cotran et al. 2005).

In tumours of spinal origin, the *neurofibromin 2* gene on chromosome 22 is commonly mutated, however supratentorial tumours more frequently display mutations in chromosome 9. Ependymomas do not have mutations that are commonly found in other gliomas such as p53. Prognosis in these tumours is dependent on the extent of surgical resection and symptoms of headaches,

nausea and vomiting are prevalent due to the dissemination of cerebro – spinal fluid.

1.3.10 Anaplastic ependymoma

These lesions are classified as WHO grade III tumours. Histological classification is difficult and controversial with one study defining the presence of any two of four parameters as indicative of anaplastic ependymoma. These parameters include mitoses, hypercellularity, endothelial proliferation and necrosis (Ho et al. 2001).

1.4 Glioma – Diagnosis

Neurological signs and symptoms

The neurological signposts most commonly associated with glioma often manifest over a period of weeks to months with the higher grade tumours presenting more rapidly than the lower grade tumours. These symptoms arise as a manifestation of the effect of the growing tumour on the surrounding tissue and include increased inter-cranial pressure, tumour invasion, obstructive hydrocephalus, tumour secretions or secondary cerebral ischemia. These initial symptoms can be nebulous and non-specific and it is not infrequent for there to be no presenting symptoms at all (Pace et al. 2017). Hence a physician needs to carefully monitor all kinds of signs and symptoms, however slight, to be sure that no patient is missed. A careful differential diagnosis must be made to be sure there are no confusions with depression, other causes of headaches and other neurological CNS disorders.

The most common symptom of a brain tumour is a headache. This is usually non-specific and occurs equally in patients both with and without increased intracranial pressure. As headache is the most common neurological complaint in the general population the importance of a thorough and far-reaching differential diagnosis cannot be over-estimated. There are several characteristics of headaches in brain tumours that can be used to enable the clinician to consider an oncological pathology when engaging in diagnosis (Forsyth and Posner 1993).

These include:

a) A daily headache on waking that rapidly improves,
b) A new headache in a middle-aged or older person,
c) A change in the pattern or severity of a pre-existing headache,
d) Exacerbation of a headache by coughing, sneezing, bending, head movement or exertion,
e) Headache with other neurological symptoms,
f) Headache with vomiting,

Aside from headaches, there are numerous other signs and symptoms caused by brain tumours. These include nausea, vomiting, confusion, memory loss and seizures and can be generalized, localizing or falsely localizing (Chandana, et al. 2008).

Generalised symptoms can include dizziness and tinnitus and papilledema caused by an increased intracranial pressure. This can also cause vision loss, change in consciousness level, loss of muscle tone, itching and shivering, yawning and hiccups. These events are usually caused by a change in

position and are known as plateau waves brought on by increased intracranial pressure (Risberg et al. 1969).

The most common localizing feature of brain tumours is seizure. Most patients with brain tumours tend to have partial seizures and they occur in around a quarter to a third of patients (Snyder et al. 1993). In many patients they are the single symptom and they are more common in low grade tumours than in high grade tumours. It is important to effectively differentiate those patients with non-convulsive epilepsy as these present in a similar manner.

Falsely localizing signs and symptoms of brain tumours include cranial nerve abnormalities, ipsilateral hemiparesis and ataxia. Again, these are all signs of increased intracranial pressure.

1.5 Imaging Modalities

1.5.1 Magnetic Resonance Imaging (MRI)

The technique of MRI is the gold standard for the imaging of brain tumours and gives the best anatomic detail of the brain (Figure 1.2). It works from the collusion of a powerful magnetic field and a radio frequency field to give the protons of water molecules a specific energy or ‘spin’ that can be detected by scanners as a contrast between different tissues in the body. There are five different tissue variables that can be used to construct images. These include ; spin density, T1 and T2 relaxation times, and flow and spectral shifts. It is through the manipulation of these parameters that contrast between the different tissue types can be obtained. The relaxation time, that is the time taken for the spin caused by the interaction of the fields to stop, can be

manipulated by specific substances known to alter these times. This property is also used to gain the maximum contrast between tissues. For brain tumours, this is most frequently done by the use of Gadolinium. The signals are so specific that very often the radiologist is able to predict a histological diagnosis.

An adaptation of traditional MRI scanning is perfusion MRI. This imaging technique is used to monitor blood flow and in the case of brain tumours can give information about tumour vascularity and may be a reliable marker of tumour grade (Dhermain 2010).

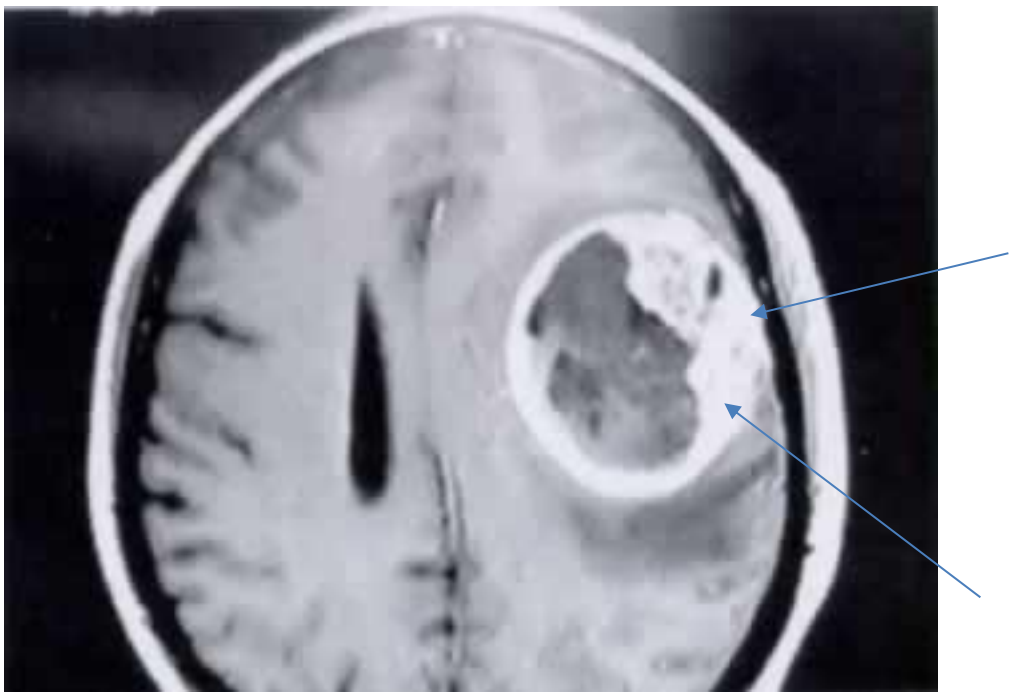


Figure 1.2. T1-weighted MRI with gadolinium contrasts showing a typical appearance of a glioblastoma multiforme, (arrows delineate the site of the tumour). Taken from http://www.neurosurgicalassociates.com.au/index.htm accessed 12/9/17.

1.5.2 Magnetic Resonance Spectroscopy (MRS)

MRS is used to measure the presence of metabolites by the analysis of biochemical spectra produced by the excitation of those metabolites. This can provide information on tumour metabolism as well as providing diagnostic information through the detection of specific patterns of change that correlate with tumour grade. For example, choline is used as a measure of cellular membrane turnover, creatinine as a measure of energy metabolism, myo-inositol signifying protein C activation, lactate to measure anaerobic metabolism and lipid as a measure of necrosis. It is also useful in measuring a patient's response to novel therapies that involve a targeted molecular strategy (Table 1.4).

Table 1.4 Table showing magnetic resonance spectroscopy changes in brain tumours.

High-grade tumours	Low-grade tumours
Elevated choline to N-acetyl aspartate ratio	Slight increase in choline
Decreased to absent N-acetyl aspartate	Slight decrease in N-acetyl aspartate
Decreased myo-inositol	Increased myo-inositol
Lipid present	No lipid
Lactate variable	

1.5.3 Computerised Tomography (CT)

Computerised tomography is an imaging technique that uses X-ray sections which are analysed by a computer to build a three dimensional image of the inside of an object from a series of two dimensional images around a central point of rotation.

The advantage CT scans have over MRI scans is that it is a superior technique for visualising calcification. Certain tumours, such as meningiomas and oligodendrogliomas, present with calcification more frequently and so CT scanning is indicated in these cases. CT scans are also used in assessing the amount of bone destruction and hyperostosis that is related to tumour growth. CT scans are used in brain tumour patients when there is a contraindication for an MRI scan (Blumenthal et al. 2015). This is usually because of the presence of medical or bio-stimulation implants such as pacemakers, vagus nerve stimulators and other ferromagnetic foreign bodies such as surgical prosthesis.

1.5.4 Positron Emission Tomography (PET)

Positron emission tomography is a nuclear medicine imaging technique that provides metabolic information with tumour imaging. A radionuclide tracer is introduced into the body on a biologically active molecule and a positron-detecting scanner detects the gamma rays that are emitted. There are a number of different substances that can be labelled with the positron-emitting isotope. These include 18-fluoro-2-deoxyglucose which images glucose metabolism. This is the most common isotope. Other isotopes include 11-carbon-methionine. Uptake of these isotopes in brain tumours is increased in

proportion with tumour grade. The tracer is phosphorylated by hexokinase that is abundant in tumour cells and cannot be metabolised from the tumour cell therefore allowing an accumulation of tracer. This means that tumour cells are highly labelled, a fact that becomes useful when looking for metastases or recurrence following resection. They are also useful in guiding stereotactic needle biopsies or for differentiating necrosis from recurrent tumour. They also have a role to play in determining response to anti-angiogenic drug treatments (Chen et al. 2009).

1.5.5 Single Photon Emission Computed Tomography (SPECT)

Single Photon Emission Computed Tomography is an imaging technique that involves injecting a gamma-emitting radioisotope and gaining two dimensional cross-sectional images of the distribution of the three dimensional isotope. This a more cost-effective, available imaging modality than PET that gives similar information. The main use of SPECT in brain tumour analysis is in differentiating tumour tissue from radiation necrosis. To this end it is used to monitor radiotherapy in brain tumour patients.

1.6 Pathology and grading

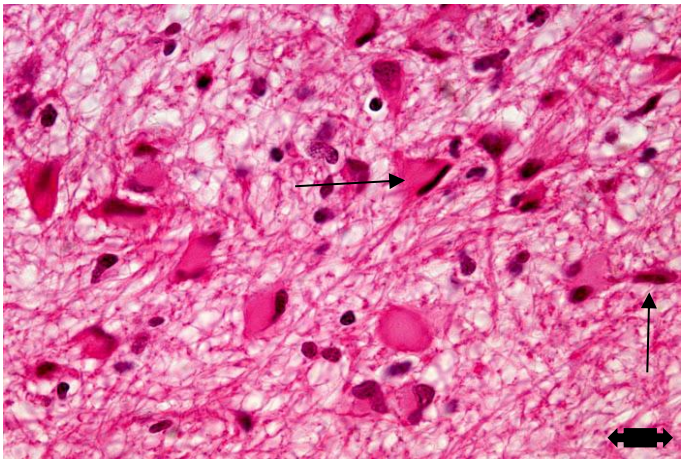
Although both clinical and neuro-imaging techniques are essential in the diagnosis of malignant glioma, a histologic examination of sample tissue remains the gold standard. The most accurate diagnosis is made when pathological studies are conducted in collaboration with a neuro-radiologist and a neurosurgeon with a full knowledge of a patient's clinical history. More recently a molecular profile of the patient's *IDH, H3 K27M* and 1p 19q deletion status has become incorporated into the 2016 WHO classification to give a

more precise definition of the tumour to aid prognosis and patient management (Louis et al. 2016). There is now for the first time an integrated diagnosis of pathology and molecular genetics.

The most commonly applied histopathologic examination on a sampled tissue is the haematoxylin and eosin (H&E) stained slide. The haematoxylin stains the nuclei of cells a dark blue colour while cytoplasmic features are counterstained with eosin to produce a reddish colour. The resulting stain allows the histopathologist to describe microscopic morphologic features of the neoplasm that correlate with biological behaviour and prognosis. This has allowed varying grading criteria to be established, the most commonly accepted of which is the World Health Organisation (WHO) classification system (Louis et al. 2016). The system is based on the histological appearance of four criteria that are related to patient survival, namely nuclear atypia, mitoses, endothelial proliferation and necrosis.

The WHO classification divides infiltrating gliomas, (grades II, III and IV) from non-infiltrating gliomas, (grade I) (Louis et al. 2016). Of the infiltrating gliomas these can be distinguished as astrocytomas, oligodendrogliomas and oligoastrocytomas based on the morphologic criteria of cell shape, cytoplasmic appearance and nuclei characteristics. This appearance is then combined with information on infiltrative capacity to give a final tumour grade. For example, an infiltrative astrocytoma is diagnosed when astrocytic tumour cells have been shown to invade CNS parenchyma. Astrocytomas in general have elongated nuclei that are hyperchromic and irregular and lacking in nucleoli and perinuclear halos (See figure 1.3).

A.



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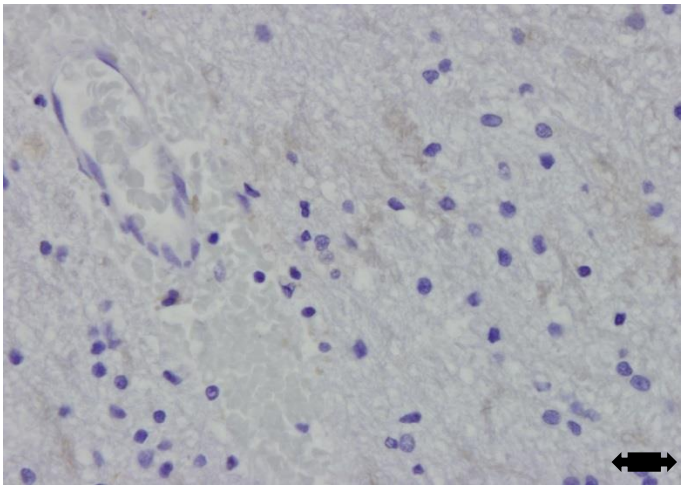
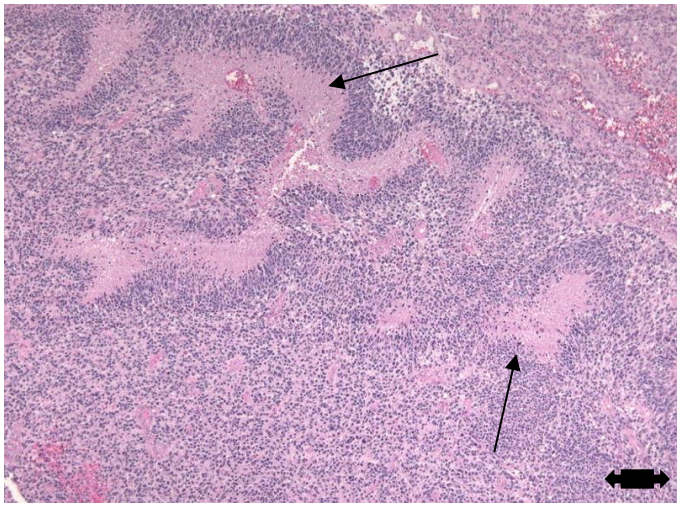


Figure. 1.3 (A) A photograph showing Astrocytoma cells displaying elongated nuclei (arrows) compared with (B) normal brain tissue (taken from (A) http://www.microscopyu.com/galleries/pathology/images/astrocytoma40x03.jpg on 18/7/11 and (B) original photograph. Black scale bar represents 20 microns.

Gemistocytic subtypes have pink cytoplasm whilst fibrillary subtypes have only a minimal cytoplasm although fibrillary subtypes are not included in the current WHO classification. Differentiation between grade II and grade III subtypes is dependent upon mitotic activity. Classification of a grade III astrocytoma is predicated upon the appearance of more than one mitotic

figure that correlates with decreased survival data (Giannini et al. 1999b). The number of mitoses identified is reliant upon the sample size of the tissue and the number of sections examined. Studies have shown that the examination of fifty 400X field allows the detection of mitoses with 92% specificity (Coons and Pearl 1998). In contrast with grade II astrocytoma the grade III anaplastic astrocytoma has increased cellularity and proliferation as well as greater nuclear polymorphism and atypia. A diagnosis of glioblastoma multiforme (WHO Grade IV), the highest grade of infiltrating astrocytoma, is dependent upon the presence of microvascular hyperplasia or necrosis which is often pseudopalisading in addition to the previous features of astrocytoma mentioned (see figure 1.4. Arrows highlight the necrotic areas)

A.



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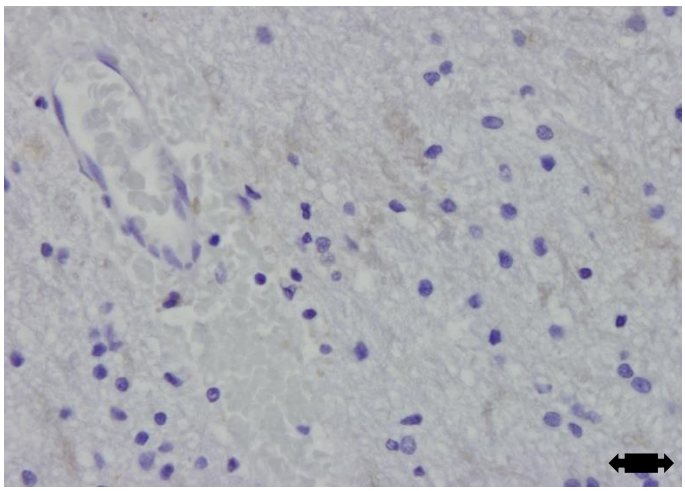
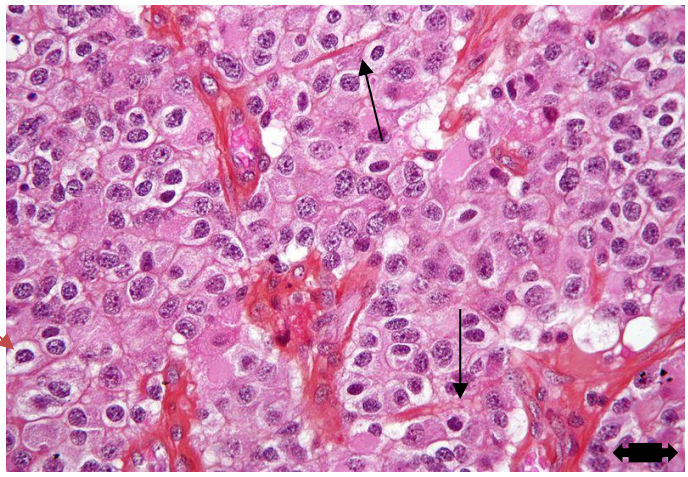


Figure 1.4. (A) A photograph showing GBM with arrows highlighting areas of pseudopalisading necrosis (Taken from wikipedia commons accessed 18/7/11) compared with (B) normal brain (original photograph). Black scale bar for A ⟷ represents 200 microns and for B represents 20 microns.

There is often a difficulty in differentiating high grade astrocytomas from oligodendrogliomas and mixed oligoastrocytomas. Oligodendroglioma cells have regular, spherical nuclei that display little variation from each other sometimes with some perinuclear cytoplasmic clearing. There is also

occasionally evidence of cortical involvement, calcification and branching capillaries (See figure 1.5)

A.



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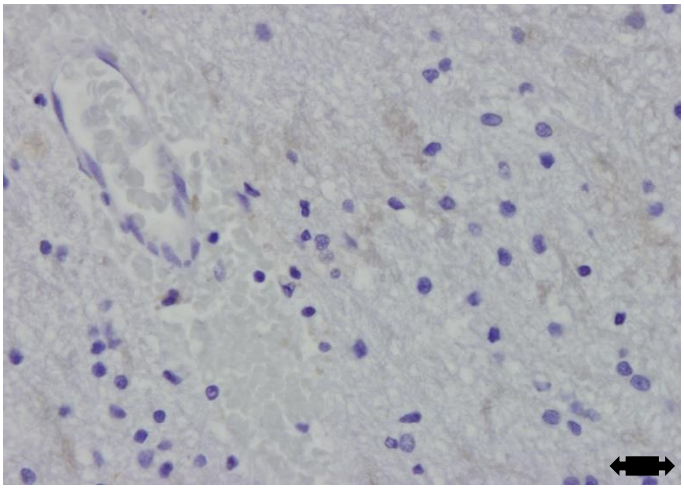
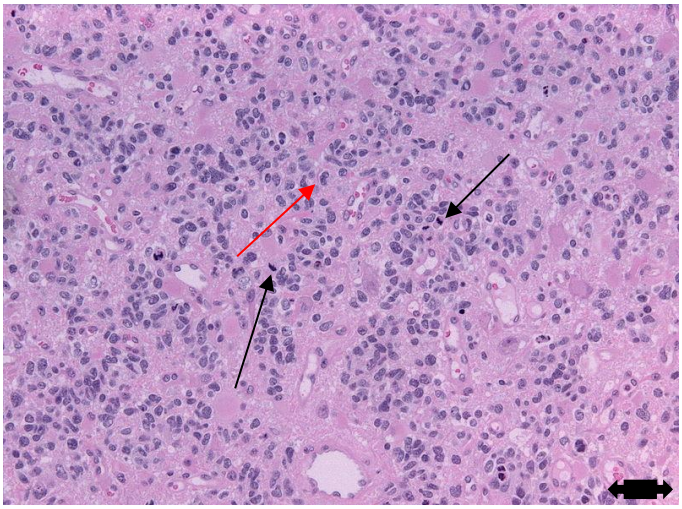


Figure 1.5 A photograph showing a section from a post-operative oligodendroglioma patient with black arrows highlighting branching capillaries and red arrow highlighting fried egg appearance of cells (Image provided by Nephron from Wikipedia commons on 18/7/11) compared with (B) normal brain (original photograph). Black scale bar ⟷ represents 20 microns.

To differentiate between Grade II and Grade III oligodendrogliomas, attention must be given to the amount of vascular hyperplasia, necrosis and the number of mitoses present with a threshold of > 6 mitoses per high power field identified as the critical cut-off in survival analysis (Giannini et al. 2001).

Oligoastrocytomas (see figure 1.6) are comprised of distinct regions of oligodendroglial and astrocytic components.

A.



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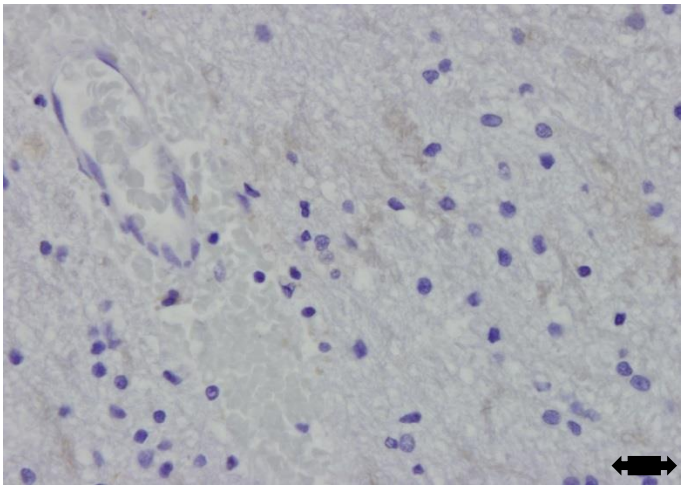


Fig 1.6 (A) A photograph showing a mixed glioma (anaplastic oligoastrocytoma) with abundant mitotic figures (black arrows) and nuclear pleomorphism (red arrow) (HE stain, x10 magnification), (provided by Jensflorin from Wikipedia Commons accessed 18/7/11 compared with (B) normal brain (original photograph). Black scale bar ⟷ represents 100 microns for A and 20 microns for B.

There has been much debate about the amount of the minimum percentage requirement of each component for a diagnosis to be made. A wide-ranging study of diagnostic criteria recommended that the presence of a single 100X field filled with an oligodendroglioma component could be used as a threshold for mixed oligoastrocytomas (Coons, et al.1997). The WHO recommendations describe the requirement of 'features of anaplasia' to be present in either the oligodendroglial component or the astrocytic component for a diagnosis to be made (Louis, et al 2016). There is some evidence to suggest that the presence of necrosis in mixed oligoastrocytomas has bearing on survival and that those with necrosis and therefore a shortened survival expectancy should be classified as a grade IV tumour (Miller et al. 2006).

Sometimes it is necessary to collect a biopsy for a frozen section diagnosis to guide the neurosurgeon at the time of the operation to their next course of action. A frozen section is a sub-optimal technique for the diagnosis of an infiltrating glioma. Although features such as cellular density, nuclear anaplasia, mitotic activity, microvascular hyperplasia and necrosis can be readily determined from a frozen section other distinguishing diagnostic features such as perinuclear halos, chromatin pattern and nuclear regularity are more difficult to discern with confidence. There is also a strong possibility that the freezing process will introduce artefacts. These are usually manifested as increased hyperchromatic nuclei (Burger and Vogel 1978). However, most studies show a high degree of correlation (85%) between frozen section diagnosis and final histological diagnosis (Brainard et al. 1997).

Immunohistochemistry can be used in brain tumour diagnosis. It is used to differentiate between tumour cells from GBM, metastatic carcinoma,

melanoma and primary CNS lymphoma (Dunbar and Yachnis 2010). The distinguishing protein is glial fibrillary acidic protein (GFAP). This is a filamentous protein expressed by normal and malignant glial cells. There is a high specificity for this stain with some studies quoting 100% expression by the tumour cells of astrocytic neoplasms (Cosgrove et al. 1989). As well as being an astrocytic marker both gemistocytes and gliofibrillary oligodendrocytes of oligodendrogliomas have also been shown to stain for this marker, (Kros et al. 1990).

O-6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that reverses the cross-linking of DNA by chemotherapeutic agents. The expression level is determined by the methylation status of the MGMT gene's promoter region which can silence the gene and produce a low level of protein expression (Riemenshneider, et al. 2010). An improved response to chemotherapeutic agents is linked to epigenetic silencing (Esteller 2000), therefore prognostic information can be obtained by testing the MGMT expression level of GBM patients.

1.7 Prognosis

There are many prognostic indicators for patients diagnosed with gliomas. This information comes from clinical trials and population registry data. Care must be taken when reviewing this data as most patients do not enter clinical trials and so the results may not be representative of the general population of patients with gliomas. This variability comes from differences within histological diagnoses between neuropathologists. Prognostic indicators for gliomas are taken from the following list: Histological diagnosis, grade of tumour, patient

age, patient race, patient sex, extent of resection, location of lesion, radiation therapy and chemotherapy taken (Curran et al. 1993 ; Davis et al. 1998). The single most significant prognostic factor in univariate and multivariate analyses has been patient age with patients less than fifty years old at the time of diagnosis having a better prognosis than older patients. This correlation was reproducible throughout the age groups in a linear fashion (Ohgaki & Kleihues 2007) as shown using a Kaplan-Meier curve, (see figure 1.7). This is a plot of the survival of a group of patients, in this case, categorised into age groupings over a period of time. There is 100% survival at time 0, the date of diagnosis, and this figure, called the cumulative survival, decreases as patients die.

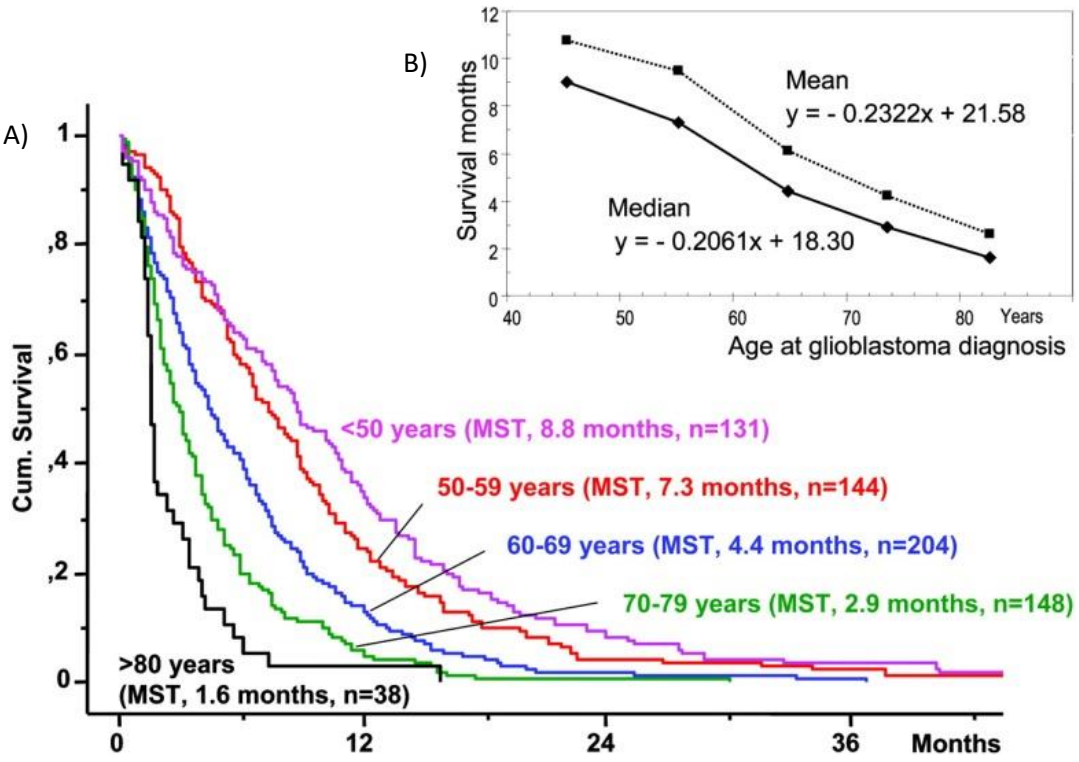


Figure 1.7. A) Kaplan-Meier curves showing that younger age of patients with glioblastoma is predictive for longer survival (log-rank test: <50 years *versus* >50 years, *P* < 0.001; 50 to 59 years *versus* 60 to 69 years, *P* < 0.001; 60 to 69 years *versus* 70 to 79 years, *P* < 0.001; 70 to 79 years *versus* >80 years; *P* = 0.0261). B) On the basis of these data, formulae were established to calculate the median and mean survival time from the date of glioblastoma diagnosis (Taken from Ohgaki et al. 2007) Accessed 18/7/11)

There has been a variation in response to therapy and there is a similar median survival across therapies. In a meta-analysis of randomised clinical trials the overall survival rate of high grade glioma was 40% at 1 year and 46% after combined radiotherapy and chemotherapy (Afra et al. 2002). The Karnofsky performance scale is a patient assessment tool used by clinicians to gauge a patient's functional status in carrying out the activities of daily living. It is

important to continually assess patients to monitor deterioration and/or the effectiveness of treatment regimens (see table 1.5). There is also a strong correlation of survival with Karnofsky scale performance (Curran et al. 1993). These results may be skewed however as most clinical trials only accept patients with better Karnofsky scores onto their treatment regimen leaving those generally older patients with poorer scores with palliative treatment only.

Table 1.5 Table showing Karnofsky performance status scale definitions rating (%) criteria

Able to carry on normal activity and to work; no special care needed.	100	Normal no complaints; no evidence of disease.
	90	Able to carry on normal activity; minor signs or symptoms of disease.
	80	Normal activity with effort; some signs or symptoms of disease.
Unable to work; able to live at home and care for most personal needs; varying amount of assistance needed.	70	Cares for self; unable to carry on normal activity or to do active work.
	60	Requires occasional assistance, but is able to care for most of his personal needs.
	50	Requires considerable assistance and frequent medical care.
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly.	40	Disabled; requires special care and assistance.
	30	Severely disabled; hospital admission is indicated although death not imminent.
	20	Very sick; hospital admission necessary; active supportive treatment necessary.
	10	Moribund; fatal processes progressing rapidly.
	0	Dead

Several studies have identified the role of the genetic changes associated with glioma previously mentioned and their relationship to survival although some such as the *TP53* mutation have inconclusive data (Ohgaki & Kleihues 2007).

1.8 Glioma – Therapy

1.8.1 Surgery

Surgical intervention is essential in the therapy of brain tumours, both in the necessity for the collection of tissue for diagnosis, to relieve the symptoms of intracranial pressure and to improve outcome by maximal surgical resection. There is strong evidence of an improved prognosis correlated with complete tumour resection (Stummer et al. 2011). This has been further improved following the use of techniques such as cortical mapping, stereotactic volumetric biopsies and resections as well as the use of robotics, lasers and aspirators (Arata et al. 2011).

Before surgery commences, it is essential for the patient to undergo a complete pre-operative assessment using the imaging modalities of MRI and CT as discussed previously and also taking into account the patient's history and physical examination. Magnetic resonance imaging provides information of tumour location and infiltration as well as the surrounding normal neurovascular structures. This is provided in sagittal, coronal and axial dimensions thereby imaging the tumour in three dimensions. Functional information is also provided by using the focal disturbance of cerebral blood flow following repetitive actions performed by the patient. This allows the surgeon to take into account the location of motor, sensory and language

cortex in relation to the tumour. Positron emission tomography scans, whilst not used routinely in glioma practice may be utilised preoperatively to identify the areas of uptake of the isotopes used guiding the biopsy to the most active areas.

There are numerous devices currently available to aid in the localisation of tumour tissue. These include fixed frame stereotactic devices that use computer programs to delineate the coordinates of the area required for biopsy. This is augmented with ultrasound and optical microscopes that enable surgeons to identify areas of tumour. Post-operatively the surgeon can use MRI imaging to ensure a complete as possible removal of the tumour has taken place. Stereotactic biopsies are considered for tumours that are buried deep within the cerebellum or for when the patient has a serious medical illness. The frame is applied to the patient under a local anaesthetic. The co-ordinates of the site are applied from information given by the MRI scan and a burr-hole is made and biopsy needle used to take the sample. Complications can arise from haemorrhage especially in vascular lesions such as glioblastoma. Sometimes an inadequate sample is taken and a diagnosis cannot be made or the diagnosis does not agree with the diagnosis from the full resection. Usually, the poor biopsy sample underestimates the grade of the tumour however diagnoses can be corrected using clinical and imaging information in conjunction with the biopsy (Quinn et al. 2011). Image-guided surgery using CT and MRI scans helps the surgeon conduct a safer and more successful operation (Amin et al. 2011).

1.8.2 Radiotherapy

The role of radiotherapy in the treatment of any cancer is to affect actively dividing cells and bringing about their death through damage to DNA, cellular organelles and cell membranes. This generates free radicals that cause DNA cross links and damage to nucleotides, the cumulative effect of which is to induce apoptosis. This is done in order to stop the spread of the tumour without damaging normal tissue. In glioma therapy, a balance is sought to supply the maximum amount of ionising radiation to the target tissue whilst excluding normal brain tissue from its harmful effects. The radiation is usually given in fractionated doses that allow a recovery time in between. Image-guided techniques allow for an accurate targeting of the tumour whilst avoiding damage to the surrounding tissue. Some factors such as cellular hypoxia and intrinsic cellular resistance reduce the efficacy of radiotherapy whilst giving radiotherapy in pulsed doses has been shown to reduce damage to normal brain tissue (Park et al. 2011).

Radiation is given as a source of photons from a cobalt source and can be given palliatively as a single treatment or it can be given radically in a schedule of 25-30 treatments. Whilst it cannot ultimately cure a tumour it can prolong life expectancy through tumour retardation in patients suffering from low-grade glioma.

External beam radiotherapy is the most commonly used technique in the treatment of brain tumours. Beams are directed to a specific three dimensional target assisted by CT and MRI imaging modalities to give a uniform dosage to a defined area. This treatment is then repeated multiple

times until the course is finished. Patient immobilisation is essential to ensure the correct area is treated. Greater precision can be found when using stereotactic radiotherapy. This involves the use of a multi-headed cobalt unit that can deliver radiation to a greater volume. This is known as gamma knife radiation therapy. There is little evidence, however, that stereotactic radiation offers a survival advantage over other techniques (Clavier et al. 2010). Another technique for delivering a dose of radiation direct to the tumour site is interstitial radiotherapy. This involves implanting a high dosage radioactive source, usually I^{125} or Ir^{192}, into the tumour placed in catheters around the site to ensure a uniform distribution. These techniques are again as yet unproven (Rainov and Heidecke 2011).

There are numerous complications to cranial radiotherapy which can be described as acute, early delayed or late delayed. Acute complications are usually associated with oedema from tissue damage and present as headaches and neurological deficit. Oedema is usually treated with corticosteroids. Early delayed complications arise from six weeks to six months from treatment. This is usually a neurological impairment resulting from demyelination of the neuronal sheath. It is a reversible phenomenon that can also be treated with corticosteroids. Late delayed toxicities arise years after radiotherapy treatment and are the result of necrosis of white matter. Clinically, this is indistinguishable from tumour progression and does not respond to treatment with corticosteroids. Demyelination is endemic as well as coagulative necrosis and these are diagnostic signs of radiation toxicity. Dementia is also sometimes an effect of radiotherapy as well as neoplasia.

Perversely a glioma can be induced from treatment with radiotherapy usually for treatment for lesions of the pituitary gland (Ron et al. 1988).

1.8.3 Blood-brain barrier (BBB)

The blood-brain barrier is comprised of brain capillary endothelial cells, the basement membrane and astrocytic feet. The endothelial cells of brain capillaries have sealed ‘tight junctions’ in which there are strands composed of trans-membrane proteins. The efficiency of the seal between the cells is proportional to the number of strands in the cells. The function of the BBB is to limit free transport of solutes between the interstitial fluid and blood. The endothelial monolayer of the BBB contains mechanisms of transport for different substances, some of which are located on the luminal side and some located on the abluminal side of the capillary. There are transporters for glucose, amino acids, vitamins and receptors that transport proteins across the endothelium from the direction of the blood to the brain. Toxic products produced by brain metabolism are transported in the reverse direction with the transport process beginning at the abluminal membrane (see figure 1.8).

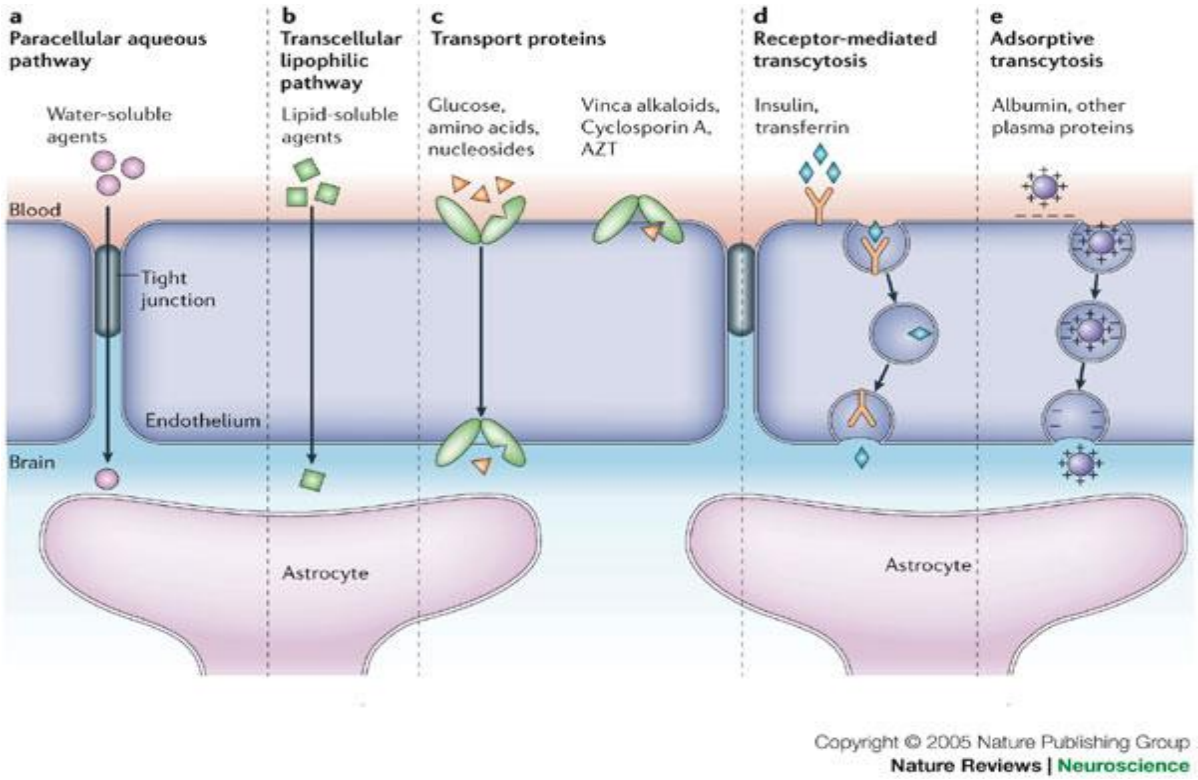


Figure 1.8. Diagram showing Astrocyte–endothelial interactions at the blood–brain barrier (Taken from Abbott NJ *et al.* 2006) accessed 21/7/11).

The BBB is the major barrier to effective treatment of glioma with chemotherapeutic agents. Although some forms of glioma are associated with BBB breakdown the tumour cells are regularly situated away from the site of disruption and are therefore inadequately exposed to chemotherapy. Research has been undertaken to elucidate the effect of blood-brain barrier disruption (BBBD) on chemotherapy efficacy (Doolittle et al. 2000). Blood-brain barrier disruption was performed by the insertion of a trans-femoral intra-arterial catheter into an internal carotid artery (ICA) or in a vertebral artery at depending on tumour location and warmed mannitol was administered for thirty seconds. This has the effect of osmotically opening the BBB allowing chemotherapeutic agents to arrive at the tumour site in a

greater concentration. This study showed improvements in efficacy with 79% of patients achieving stable disease with a low incidence of catheter related complications.

Another treatment designed to circumvent BBB issues has been the use of biodegradable wafers impregnated with chemotherapeutic agents. The trial conducted by Westphal et al. (2003) found an improvement in median survival of 2.3 months in patients using wafers compared to those that did not. There were toxicities seen in the study including seizures, haemorrhages, cysts and wound infections.

1.8.4 Chemotherapy

In inducing cancer cell death chemotherapeutic agents exert their effects through a variety of mechanisms (see figure 1.9).

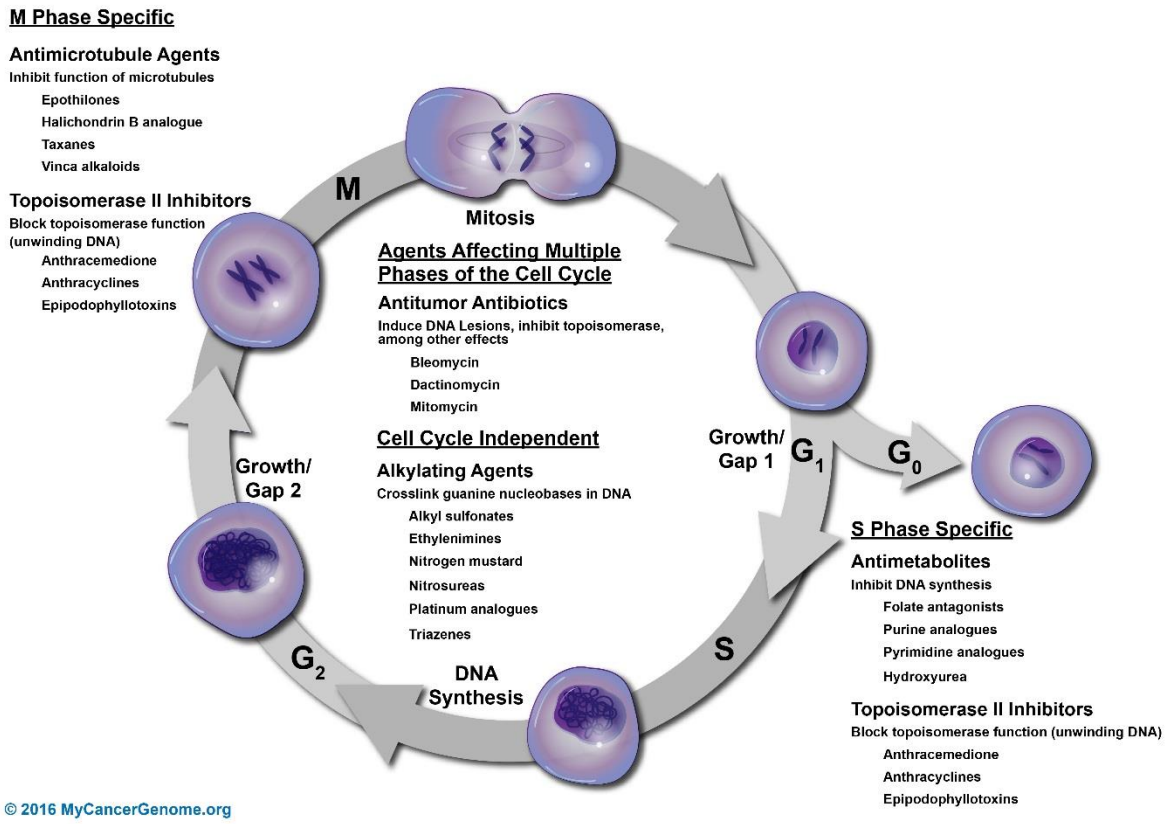


Figure 1.9 A figure showing both the cell-cycle dependent and independent mechanisms of chemotherapeutic agents. Taken from [https://www.mycancergenome.org/content/molecular-medicine/pathways/cytotoxic-chemotherapy-mechanisms-of-action accessed 21/10/2016](https://www.mycancergenome.org/content/molecular-medicine/pathways/cytotoxic-chemotherapy-mechanisms-of-action)

Temozolomide is the most commonly prescribed chemotherapeutic agent for GBM and anaplastic astrocytoma (Yung 2000). It works by inducing DNA alkylation at multiple DNA locations producing single and double strand lesions. Temozolomide also suppresses MGMT levels, which reduces a resistance to DNA damage. Procarbazine is another alkylating agent that inhibits nucleic acid and protein synthesis. There are several side-effects connected to this treatment including rashes, nausea and vomiting. Vincristine

is an agent that depolymerises microtubulin formation that arrests the cell cycle at mitosis. It is effective with high and low grade gliomas, albeit less sensitive against low grade tumours, and has a neurological toxicity (Franceschi et al. 2017).

1.8.5 Other treatment options

As discussed above, the blood-brain barrier is a major obstruction for the delivery of chemotherapeutic drugs. Other treatment modalities are being explored including nanotechnology, cannabinoids, oncolytic viruses, genetic and molecularly targeted therapy and vaccines. Drug delivery nanosystems are a strategy being developed to overcome the barrier of the BBB which incorporate the protection of therapeutic agents, their bio-distribution and therapeutic index (Roger, et al. 2011).

1.9 Cytokines

1.9.1 General aspects

Cytokines are specialised proteins involved in the regulation of the function of cells of the innate and adaptive immune system and are secreted by those cells in response to microbial invasion as well as other antigens. They are implicated in the inflammatory and immune responses by stimulating the growth and differentiation of lymphocytes and in the activation of cytotoxic effector cells. They also play an important role in haemopoiesis and are used clinically to stimulate or inhibit inflammation (Christofides, et al. 2015).

Cytokines have a diverse structure, yet share many properties. They include the following;

1. Cytokine secretion is brief. There is no long-term storage of cytokines and synthesis is *de novo* through signal transduction pathways leading to cellular activation and gene transcription. Most cytokine mRNAs are inherently unstable and therefore produce a transient, short lived burst of cytokine production. This production is controlled by post-translational modification using proteolytic processing resulting in the release of an active product from an inactive precursor (Ehrentraut and Colgan 2012).
2. Cytokines display the properties of pleiotropy and redundancy (Ozaki and Leonard 2002). Pleiotropy is the effect of a single cytokine on more than one cell type and is a disadvantage when considering therapeutic use as the number of unwanted side effects could be unmanageable. Redundancy is the property of multiple cytokines having the same effect and this also has therapeutic implications as the antagonist of one cytokine can be circumvented by others.
3. Cytokines can stimulate or antagonise the production of other cytokines. This can lead to an over production through a cytokine cascade or it could lead to the mutual antagonism of different cytokines to cancel each other out making a measured and predictable response difficult to achieve.
4. Cytokines can act in an autocrine (same cell), paracrine (nearby cell) or endocrine (distant from site of production) fashion. Some cytokines have both local and systemic effects, eg. Tumour necrosis factor (TNF) (Locksley et al. 2001).
5. Cytokines act as ligands and bind to cell receptors with great affinity usually having a dissociation constant greater than the antibody-antigen dissociation constant. This impacts on cytokine and cytokine receptor

production by requiring only a small amount of cytokine to exert its effect on cells.

6. Cytokine receptors are expressed by signals from the external environment which influences the rate of response of those cells to the specific cytokine. Therefore, there is a tendency for stimulated B and T cells of the adaptive immune system to increase the expression of cytokine receptors thereby retaining the specificity of the immune response. This does not indicate however that the cytokines themselves are specific and the same receptor can be likewise blocked by the ligand providing a negative feedback loop as well as a positive amplification loop.
7. The change in gene expression of the target cells usually imparts a new function on the target cell. Examples of this include the isotype switching function in B cells and the production of T_H1 and T_H2 subsets in helper T cells. Some cytokines such as TNF induces apoptosis without gene transcription and others called chemokines affect chemotaxis through changes in cellular adhesion molecule production.
8. There are feedback mechanisms that inhibit cytokine production and they serve to regulate cellular responses which involve the stimulation of genes that code for inhibitors of the receptors or the downstream signalling pathways activated by the receptors. These include decoy receptors, kinase inhibitors and transcription factor inhibitors (O'Shea and Murray 2008).

Cytokines can be classified into groups according to function (Abbas 2010). These are as mediators of the innate immune system, mediators of the adaptive immune system and as mediators of haemopoiesis. The cytokines of

the innate and adaptive immune system are generally produced by different cells but the distinction is not absolute and overlapping of function does occur.

The regulatory cytokines of the innate immune system are derived from mononuclear phagocytes when responding to infection. Binding occurs on toll-like receptors (TLRs) to molecules derived from pathogens such as bacterial lipopolysaccharide (LPS) and dsRNA from viruses. These can be either cell surface receptors or in the endosomes of macrophages. Cytokines derived in this way include TNF, IL-1, IL-12 and IFN-γ. These cytokines can also be secreted by T-cell stimulated macrophages. These cytokines act on endothelial cells and leukocytes to stimulate the inflammatory response.

The regulatory cytokines of the adaptive immune system are derived from T-lymphocytes responding to the specific recognition of antigen. These cytokines include IL-2, IL-4, IL-5 and IFN-γ. They are implicated in the regulation of lymphocyte maturation and growth and thus have an intrinsic role in the activation of T-cell-dependent immune responses. Other cytokines derived from T-cells are involved in the recruitment and activation of neutrophils and eosinophils. These enable the activation of foreign antigen elimination (Nemeth and Mocsai 2016).

All cytokine receptors (see figure 1.11) have a trans-membrane protein via which external ligand binding and internal signal transduction and gene transcription take place. The signalling pathways are usually initiated by the juxtaposition of the cytoplasmic domains of receptor molecules. Cytokine receptors are classified according to structural homologies of their extra-

cellular domains and the intracellular signal transduction pathways and can be divided into families.

Type 1 receptors contain at least one domain with two conserved pairs of cysteine residues and a membrane proximal sequence of tryptophan-serine-X-tryptophan-serine (WSXWS) in which X is degenerate. These are the receptors that bind type 1 cytokines in which the cytokines tertiary structure consists of four α-helical strands. The specificity of individual cytokine binding is maintained by the variable amino acid residues in the receptor. The receptors usually differ in the ligand binding chains and share the signal transduction chain. All type 1 receptors use the Jak-STAT signal transduction pathway. Type II receptors do not have the WSXWS, use the Jak-STAT pathway and have a single polypeptide binding chain and a single signal transduction chain (Schwartz and Bonelli et al. 2016).

The toll-like receptor is a feature of the interleukin-1 family of receptors and is also called the IL-1 receptor. This utilises the Interleukin-1 receptor – associated kinase (IRAK) family of kinases to induce gene transcription. The cytokines that utilise this family of receptors include IL-1 and IL-18. The TNF receptor family consists of proteins that have conserved trimeric cysteine-rich extracellular domains and utilise either the TNF receptor associated factor (TRAF) to activate gene transcription or the ‘death domain’ family adapter (FADD) to induce apoptosis (Baker and Reddy 1996).

Seven-trans-membrane α-helical receptors cross the membrane several times are sometimes called G-receptors because they utilise guanine triphosphate

(GTP) binding proteins. These are chemokine receptors and are involved in chemotaxis and other inflammatory responses.

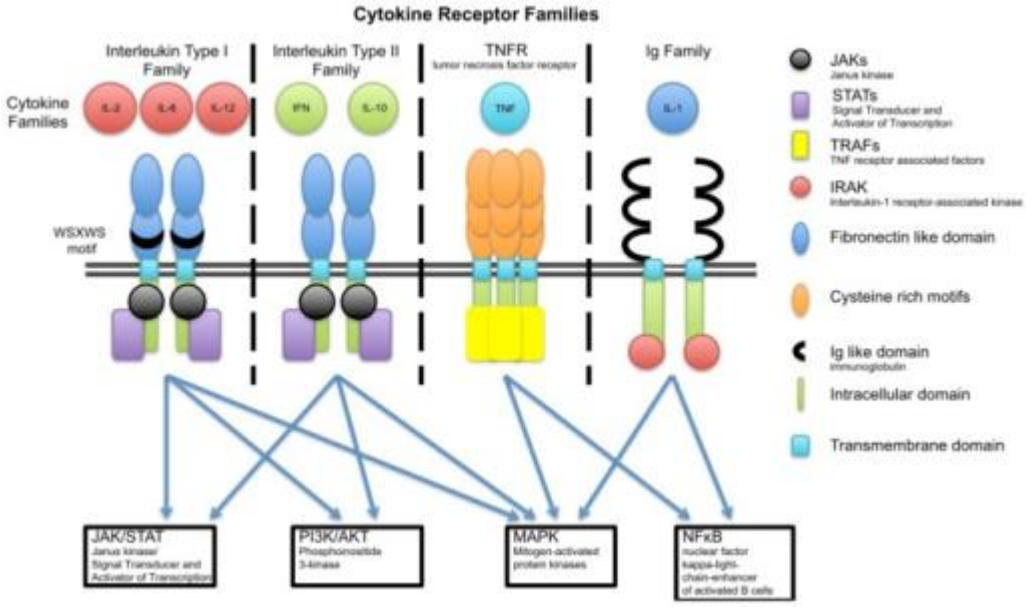


Figure 1.10 Diagram showing the structure of cytokine receptors (Taken from Hanel et al. 2013).

Cell-mediated immunity (CMI) serves as part of the adaptive immune system and is an effector function of T-lymphocytes in action against invading organisms that reside and reproduce inside host phagocytes. Mosmann et al. (1986) developed a model of CD4+ T helper cells divided into two subsets called Th1 and Th2 based on the expression of cytokines as well as helper function. Later experiments described by Coffman (2006) showed Th1 cells secreting IL-2, IL-3, TNF-α and IFN-γ whilst Th2 cells secreted mainly IL-4, IL-5 and IL-13. This differentiation was also associated with function with the Th1 cytokines controlling cell-mediated functions like macrophage activation and Th2 cytokines stimulating humoral immunity by activating B-cells and initiating class switching. Th1 differentiation usually occurs as a response to

marcophages affecting microbes and those that activate natural killer T cells. Th2 differentiation occurs in response to infection by parasitic organisms such as helminths as well as allergens that cause little macrophage activation. A further function of the differentiated Th subtype is the ability to reinforce the production and expansion of that subtype and the ability to inhibit the production of the other subtype. Each Th subset therefore can produce an individual effector function for any particular immunogen and can be divided into proinflammatory (Th1) or anti-inflammatory (Th2) responses (see figure 1.12).

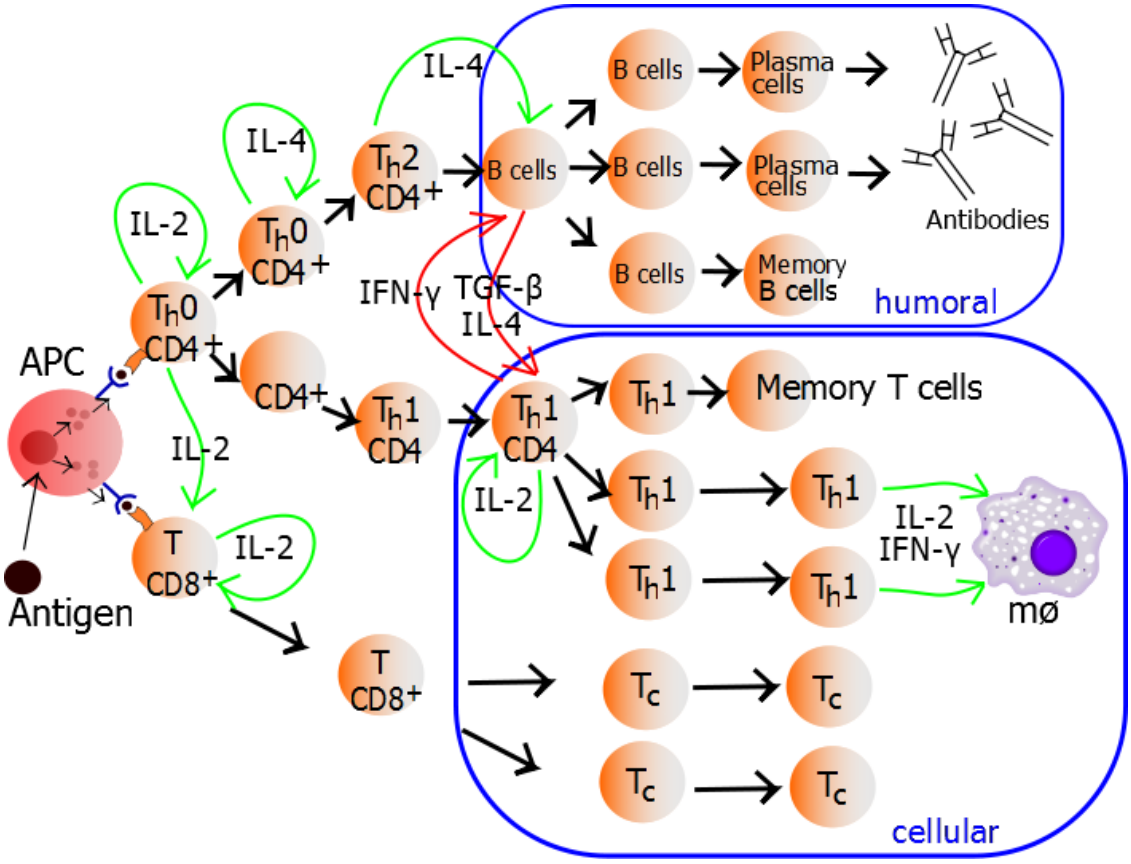


Figure 1.11. Diagram showing the development of lymphocyte subsets (Taken from Rang, 2003).

In figure 1.12 an antigen is ingested and processed by an antigen presenting cell (APC) such as a macrophage with the peptide antigen being presented on the surface of the cell via the multi-histocompatibility complex (MHC). Lymphocytes are initially differentiated into their subtypes (CD4 positive or CD8 positive) by cytokine release from APCs such as macrophages and dendritic cells. Although these cells are normally part of the innate immune system, they connect to the adaptive immune system by secreting the specific cytokines that will trigger differentiation towards a Th1 or Th2 response. Yoshimoto et al. (1998) demonstrated that proteins obtained from the APC such as IL-12 and IL-18 are involved in the development of a Th1 cell response, which is self-perpetuating because these cytokines up-regulate their reciprocal receptors on other Th1 cells. Th2 differentiation is through IL-4 after the naïve CD4+ T cell has bound to APCs.

1.9.2 Role of cytokines in immune surveillance in cancer

Cytokines play an important biochemical and physiological role in modulating the immune responses to tumour growth and development. They can influence the growth and spread of tumours through the recruitment of pro-inflammatory cell types that can also affect angiogenesis. They can also act more directly by promoting or inhibiting tumour cell growth. The monitoring and editing of the tumour by the immune system is termed tumour immune surveillance (TIS) and the evasion by the tumour of an immune response is termed tumour immune escape (TIE). Cytokines have variable effects and influence in tumourigenesis and growth (Libetta, et al. 2016 ; Mullen and Gonzalez-Perez 2016).

Interferon gamma (IFN-γ) is an essential cytokine in promoting a formidable anti-tumour response. This cytokine plays both a protective role as well as a pathologic role (Boehm et al. 1997). The IFN-γ receptor is ubiquitous and interacts with the cytokine after being produced by natural killer (NK) cells initially and later by CD4+ and CD8+ T cells. This means that IFN-γ is involved in both innate and adaptive immune responses and has roles in the production of Th1 responses, cytotoxic T cell responses and in the activation of macrophages. Mice lacking the IFN-γ receptor subunit 1 have 20 times more sensitivity towards developing tumours (Bach et al. 1997). Endogenous IFN-γ also protects the host from *de novo* tumour formation and acts alongside granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-12 in tumour immune responses (Ikeda et al. 2002).

IL-12 is secreted from APCs and it demonstrates anti-angiogenic activity when acting with IFN-γ by up-regulating MHC class I and II expression on tumour cells as well as the activation of NK cells, CD8+ CTL and the production of interferon-inducible protein 10 (IP-10) (Nastala et al. 1994). It has a significant anti-tumour response towards experimental as well as spontaneous metastases (Mu et al. 1995).

GM-CSF has been shown to increase cell-specific cytotoxicity towards autologous tumour targets (Soiffer et al. 2003).This cytokine is usually involved in haemopoietic progenitor growth and differentiation yet it also has a role in the maintenance of a durable and powerful specific anti-tumour immune response.

The inflammatory response of neutrophils and other leucocytes have been implicated in the modulation of the anti-tumour response. There has been a link between inflammation and cancer whereby inflammatory components are involved in tumour promotion through their ability to respond to factors that support angiogenesis, tumour growth and tissue remodelling (Demaria et al. 2010).

Tumour necrosis factor-α (TNF-α) is produced by macrophages but also by tumour cells and it has a role in tissue destruction, recovery and remodelling. This cytokine displays both pro-and anti-tumour properties in that on the one hand it can cause necrosis in tumours, but then it also induces nuclear factor – κβ (NF-κβ) which is involved in anti-apoptotic activity (Balkwill and Joffroy 2010). TNF-α can also inhibit DNA repair and induce angiogenic factors. These schizophrenic roles could be explained by the great diversity found in the TNF receptor complexes that stimulate different transduction pathways.

Interleukin-6 (IL-6) displays pro-tumour properties. It has been shown to be involved in the stimulation of tumour growth and metastasis (Chen et al. 2016). Other cytokines such as IL-10 and transforming growth factor-β (TGF-β) are immunosuppressive cytokines that regulate effector responses through various different ways.

The tumour microenvironment contains many soluble components that contribute to local immunosuppression and evasion. Transforming growth factor – beta (TGF-β) is a major immunosuppressant that binds to cell surface receptors to form a heterodimeric complex comprised of two pairs of subunits type I and type II. This binding elicits downstream regulation of TGF-β

dependent gene expression. The role of this gene has been demonstrated by Letterio and Roberts (1998) as one of the suppression of T-cell immune responses. Mice deficient in TGF-β receptors display a gross inflammatory response resulting in death within 3-4 weeks with an aberrant expression of MHC class I and II antigens. This suppression may come from TGF-β expression and secretion by regulatory T cells (Tregs). These cells secrete increased levels of TGF-β and are involved in immunological homeostasis. Treg cells are CD4+ CD25+ Foxp3+ cells and comprise around 5-10% of circulating peripheral T lymphocytes (Nishimura et al. 2004). It has been shown that Foxp3+ expression and suppressive activity and the size of the Treg cell compartment respond to signals initiated by TGF-β. These Treg cells, amongst others, are condensed in the tumour micro-environment thereby suppressing immune responses to tumour growth (Lopez et al. 2006) and are numerically increased in different cancers (Nomura and Sakaguchi 2005).

Interleukin-10 (IL-10) is a cytokine that is produced by T-cells, B-cells, monocytes and macrophages. It has been shown to have both immunosuppressant and immunostimulatory effects (Singh et al. 2015). It can inhibit cytokine production by Th1 cells in response to APCs as well as blocking the production of co-stimulatory molecules such as CD80 and CD86. This has the effect of limiting the strength of immune cell activation (Salazar-Onfray 1999). The mechanism for such action is thought to be due, in part, to the inhibition of the maturation of dendritic cells and the subsequent down-regulation of their capacity to initiate a Th1 response. Moreover it has also been shown to be the only cytokine that has an inhibitory effect on MHC class

I antigen presentation leading researchers to veer towards its role as an immunosuppressant as its primary function. Assi et al. (2014) have demonstrated the effect of IL-10 in antigen presentation is associated with a corresponding increase in MHC class II presentation. Along with its inhibition of pro-inflammatory cytokines such as IL-2, IFN-γ in both lymphocytes and monocytes, it has also been described as present in a wide range of tumour biopsies such as melanoma, renal carcinoma and ovarian tumours as well as metastatic tumours.

Prolactin has been traditionally associated with mammary development and lactation. However, more recently several studies have implicated the hormone in tumourigenesis through disruption of the JAK-STAT pathway (Gorvin 2015). and its receptor in glioblastoma is being elucidated. Studies have detected the presence of intracellular prolactin, prolactin receptor (PRL-R) and hyperprolactinemia in different types of central nervous system (CNS) tumours. Van Meir,et al.(1990);Ciccarelli,et al.(2001) discovered the presence of PRL-R and hyperprolactinemia in 45.4% and 27.2% of meningiomas and 69.2% and 61.5% of schwannomas, respectively. In these studies the level of the receptor and the ligand were not correlated. Soares et al. (2007) discovered the presence of PRL-R in 39% of patients with CNS tumours and the presence of intracellular prolactin in 21.9% of those patients. There was also hyperprolactinaemia in 30% of 82 cases. Their data suggested a positive correlation with serum prolactin and intracellular prolactin. The study analysed 12 high grade, 6 low grade and 6 non-cancerous specimens. The stained specimens were graded as 1, 2 or 3 according to the intensity of staining in the nucleus,

cytoplasm or interstices using the scoring system outlined in chapter 2, section 2.4.

Leptin is a cytokine produced by adipocytes and it is associated with the satiety function and appetite regulation through interaction with the hypothalamus, (Zhang, et al. 1995). Leptin has also been demonstrated to influence cellular proliferation as well as involvement in the suppression of apoptosis in cells of breast, colorectal, prostate, pancreatic, ovarian and lung cancers (Garofalo and Surmacz 2006). All these cancers demonstrated increased serum levels of leptin. There have been no studies to date showing an increased serum level of leptin correlated with glioma malignancy, a finding supported by this study. Nevertheless, Leptin has been shown to enable tumourigenesis, angiogenesis and metastasis in these cancers (Zhou, et al. 2011) through the stimulation of the angiogenic factor VEGF (Ferla, et al. 2011) and an investigation into its expression in high and low grade glioma tissue is warranted. Riolfi, et al. (2010) found a highly significant correlation between the expression of leptin and its receptor ObR and the degree of tumour malignancy. Decreased expression of both markers was found in low grade astrocytomas and gangliogliomas compared with high grade anaplastic astrocytomas and glioblastomas. Morash, et al. (2000) reports expression of the Leptin gene and ObR in the C6 rat glioblastoma cell line and found that leptin was absent in the cytoplasm of the C6 cells, but was present in the nucleus. There has since been a limited amount of evidence to support this finding.

1.9.3 Role of cytokines in glioma

The first evidence for the secretion of protein from glioma cells was reported in 1984 by Fontana et al. (1984). This was a factor produced from a glioma cell culture that inhibited T cell proliferation and function following induction with IL-2. It was shown that this factor was an isoform of TGF-β which as well as suppression of T-cells and monocytes has been implicated in the formation of Tregs as described above. Further studies have demonstrated an *in vivo* role for TGF-β (Th3 cytokines) through immunohistochemical analysis. This study has shown that areas of tumour with greater TGF-β concentrations are negatively correlated with a lower number of infiltrating lymphocytes (Horst et al. 1992). However, blood serum levels of TGF-β have shown no difference between GBM patients and normal controls (Krzyszkowski et al. 2008).

Recent work has highlighted the role of cyclo-oxygenase type 2 (COX-2) expression in glioma which with a secondary induction of IL-10 from dendritic cells has resulted in the generation of Treg cells (Akasaki et al. 2004). Some authors report that rather than IL-10 being secreted from glioma cells, it is instead restricted to circulating monocytes and infiltrating macrophages (Samaras et al. 2007). Monocyte cultures can be functionally manipulated by the down-regulation of IL-12 and a concomitant increase in IL-10 production by secreted factors from cultured glioma cells (Zou et al. 1999a). A subset of monocyte cells has been found to suppress CD4+ and CD8+ T cells (Ostrand-Rosenberg and Sinha 2009) and is termed myeloid-derived suppressor cells (MDSC). These cells are able to pass through the blood-brain barrier into the tumour microenvironment and may be the source of systemic immunosuppression in GBM patients. Interestingly patients with GBM display

a monocytosis with increased numbers of monocytes with the MDSC phenotype.

There are various mechanisms by which malignant tumour cells may alter the production of endogenous cytokines through interaction with exogenous cytokines. These mechanisms include a change in the expression of cell surface receptors and changes in secondary messenger systems. Malignant gliomas show the most change in the alteration of cell surface receptors (Fukuyama et al. 2007) and GBM patients most frequently express Th2 and Th3 cytokines (Jackson et al. 2008). The source of the Th2 cytokine secretion, whether from peripheral or infiltrative lymphocytes or the glioma cells themselves, is still not clear.

The brain is usually thought of as an immunologically privileged site with the blood-brain barrier, no lymphatic drainage and reduced expression of HLA molecules there is opportunity for an effective immune response due to the expression of antigens by the glioma cells, thereby stimulating immune cells towards the tumour microenvironment (Yamanaka 2008). More recently, however, the view that the brain has no lymphatic drainage has been challenged by authors who have found a lymphatic vessel drainage system in the *dura mater* of mouse brains (Louveau 2015 ; Aspelund et al. 2015).

There is evidence that cytokines play a fundamental role in the expression of HLA molecules on antigen-presenting cells as well as on tumour cells (Papaetis et al. 2008). After an immune response has occurred, the continued production of cytokines is required to maintain the response. For example IL-12 promotes IFN-γ release by NK cells that channels a Th1 pro-inflammatory

response that supports cytotoxic T lymphocyte (CTL) function (Lemire et al. 2017).

Prostaglandin E2 (PGE2) is another molecule which is implicated in immune suppression in a paracrine fashion. It acts to down regulate Th1 cytokine production and up regulates Th2 cytokine production. This molecule is produced by the action of the cyclooxygenase enzymes that convert arachidonic acid to PGE2 (Echizen, et al. 2016). The increased levels of PGE2 stimulates angiogenesis and motility of tumour cells and overexpression of COX-2 is associated with increased tumour infiltration. Other work has demonstrated that media containing PGE2 enhances the immunosuppressive activities of Tregs through the induction of mature dendritic cells to overexpress IL-10, a potent immunosuppressive cytokine (Yaqub et al. 2008).

Angiogenesis is the proliferation of novel blood vessels from ones already formed. It is a complex interaction of molecular signalling events that herald the process of basement membrane degradation, the proliferation of endothelial cells, the recruitment of perivascular cells and the invasion of the adjacent stromal space (Jansen et al. 2004). The tumour vessels are highly irregular unlike normal vessels with twisted and dilated vessels of various diameters. There are more numerous branching points and shunts and the vessels contain endothelial fenestre and vesicles.. The endothelial cells themselves display an abnormal shape with overlapping growth patterns that prove to be inefficient blood transporters due to the numerous leaks caused by such structure. It has been suggested that this is the major cause of some

clinical problems experienced by tumour sufferers such as brain oedema (Eberhard 2000).

Cytokines and growth factors as well as enzymes and adhesion molecules influence the process of angiogenesis (see figure 1.13). Genetic alteration can lead to the overexpression of angiogenic factors as well as an hypoxia-induced switch to pro-angiogenic factor production from anti-angiogenic production is generated at the very early stages of tumourigenesis. The signalling pathways of angiogenesis in glioblastoma are controlled mainly by the vascular endothelial growth factor (VEGF), family of molecules and receptors (Alitalo and Carmeliet 2002), as well as others. These include angiopoietin (Reiss et al. 2005), platelet derived growth factor (PDGF) (Dunn et al. 2000), IL-8 (Gao, et al. 2010), follistatin (Shi, et al. 2016), hepatocyte growth factor (HGF) (Hartmann, et al. 2016), transforming growth factor beta (TGF-β) (Mangani, et al. 2016) and tumour necrosis factor alpha (TNF-α) (Capitao and Soares 2016).

Vascular endothelial growth factor and its receptor perform numerous activities when expressed in glioblastoma vasculature. It acts as a stimulator for the proliferation, migration and survival of endothelial cells. It is also involved in regulating the permeability of blood vessels facilitating the transport of plasma proteins. Other functions include the stimulation of nitric oxide synthase that results in the production of nitric oxide, a potent vasodilator including the stimulation of monocyte and neutrophil migration (Bouloumie et al. 1999). In conditions of hypoxia transcription of VEGF-A, one of six homologues, is up-regulated by hypoxia-inducible factor 1 (HIF-1). Moreover, mRNA stability is increased and an RNA binding protein preserves

translation which can lead to an increased production of VEGF-A protein and increased expression of the receptor (VEGFR-1) contributing to glioblastoma angiogenesis (Goldberg and Levy 2000). Another effect of hypoxia is the down regulation of thrombospondin-1 expression which is a glycoprotein involved in the inhibition of endothelial cell division (see figure 1.13).

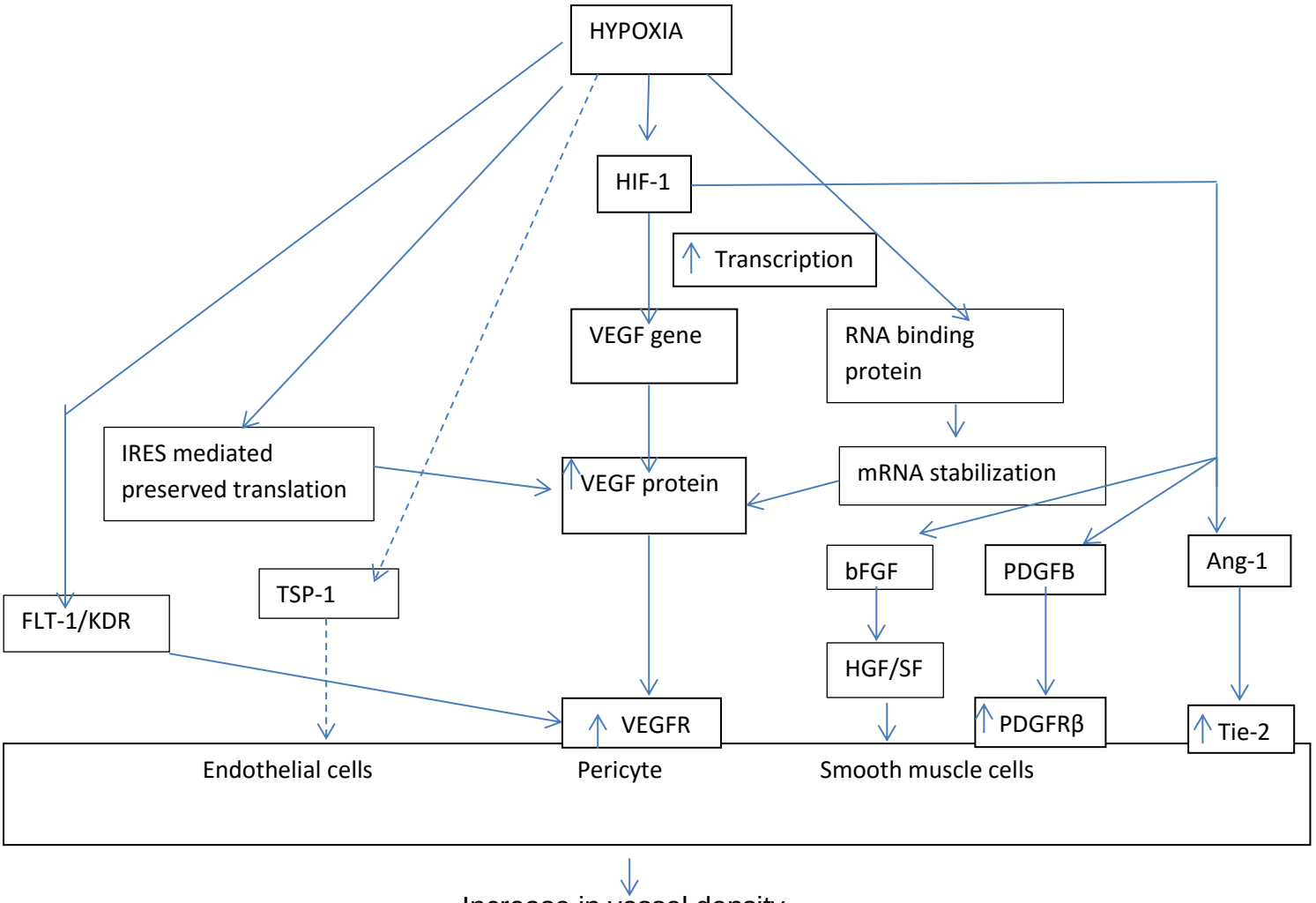


Fig 1.12 Hand drawn flow chart showing molecular events of glioblastoma angiogenesis adapted from (taken from Choudhury et al. 2010).

Figure 1.13 key

IRES – Internal ribosomal entry site, PDGF – Platelet-derived growth factor,

VEGF – Vascular endothelial growth factor, VEGFR- VEGF receptor, Ang- Angiopoietin,

TSP-1 – Thrombospondin-1, HGF/SF – Hepatocyte growth factor/scatter factor,

bFGF – Basic fibroblast growth factor.

Upregulation

Downregulation

There are three VEGF receptors known to influence angiogenesis and they include VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and 2 are associated with tumour angiogenesis while VEGFR-3 is usually associated with lymphangiogenesis (Alitalo and Carmeliet 2002). VEGFR-1 and 2 are expressed on mature vascular endothelial cells as well as circulating bone marrow derived endothelial progenitor cells. VEGFR-1 has also been found on macrophages and pericytes. The VEGF receptors transmit signals through the mitogen-activated protein kinase (MAPK) pathway and are tyrosine kinase receptors. Upon ligation with the VEGF ligand, the receptor dimerizes and becomes trans-autophosphorylated. VEGF-A is secreted by the tumour which plays a role in the migration of endothelial progenitor cells (EPCs) and haemopoietic progenitor cells to the tumour leading to vascular development as both VEGFR-2 and VEGFR-1 are expressed by EPCs and haemopoietic cells, respectively. Therefore for angiogenesis to occur both VEGFR receptors must contribute to the recruitment of endothelial and mesenchymal cells (Lyden et al. 2001).

Platelet-derived growth factor (PDGF) normally targets capillary endothelial cells, the smooth muscle cells of the vessel, glial cells and neuronal cells. PDGF also has roles in embryonic development (Andrae, Gallini et al. 2008). There are several isoforms that consist of dimerized A or B chains and these are PDGF-AA, PDGF-AB or PDGF-BB. The PDGFB molecule is up-regulated in hyperplastic tumour endothelial cells. This ligand can signal downstream transcription and up-regulation of VEGF by endothelial cells (Dunn, Heese et al. 2000).

The platelet-derived growth factor receptor beta (PDGFRβ) has been noted on tumour vasculature cells (Li, Xu 2016a) and as PDGF-BB can signal through all PDGF receptors. In turn it is able to drive tumour progression, and it has been noted to upregulate the production of VEGF-A – an important angiogenic factor (Xue, Lim et al. 2013).

Angiopoietin belongs to a family of growth factors that act in concert with VEGF in promoting neovascularisation during the formation of a tumour. Angiopoietin-1 and angiopoietin-2 bind to a receptor tyrosine kinase on the endothelium called Tie-2 (see figure 1.13). Angiopoietin-1 is secreted by normal pericytes and stromal cells and acts in a paracrine fashion with tumour cells to cause the phosphorylation of the Tie-2 receptor. This results in the stabilisation of newly formed blood vessels from the interaction of VEGF. In the absence of VEGF. Angiopoietin-2 has a regressive effect on vascularisation and promotes endothelial cell apoptosis (Reiss et al. 2005).

Activin A is a well-known pro-angiogenic factor that is inhibited by follistatin. They are both pleiotropically implicated in cellular proliferation, differentiation and apoptosis in both paracrine and autocrine fashion and follistatin has been shown to be a potent promoter of angiogenesis (Welt, Sidis et al. 2002).

Apoptosis in glioblastoma is most frequently disturbed by the activation of anti-apoptopic pathways. This is usually through the perturbation of the P13K/Akt/mTOR signal transduction pathway. However, there are other regulators of apoptosis and glioblastomas which have been shown to overexpress Fas ligand, Bcl-2 and TGF-β2, all thought to be apoptosis regulators (Choi et al. 2004).

1.9.4 Potential use of cytokines as a biomarker

In order to be an effective biomarker either blood plasma and/or serum cytokine or angiogenesis factor secretion must have both a high sensitivity (few false negatives) and a high specificity (few false positives). It must also be responsive to disease grade and progression as well as to treatment and interventions. Another consideration is that levels must be detectable at statistically significant levels using a readily available and straight forward analytical technique.

Zisakis et al. (2007) have used an ELISPOT methodology to measure Th1 and Th2 cytokine secretion from mononuclear cells as well as tumour cells. The measured cytokines were TNF-α and IFN-γ, both Th1 cytokines, and IL-4 and IL-10 from the Th2 cytokines. The results showed that Th1 cytokines, the 'immuno-reactive' cytokines, were reduced compared to normal controls whilst the Th2 cytokine levels, the immunosuppressive, were 'strongly expressed' in both lymphocytes and tumour cells.

The study by Zisakis et al (2007) is consistent with previous work that demonstrates a shift from a Th1 cytokine profile towards a Th2 type cytokine profile in the aetiology of glioma (Hao et al. 2002). It has also been shown that mononuclear cells from glioma patients are less responsive to mitogenic stimulation of Th1 cytokines (Urbani et al. 1995). For the Th2 cytokines, the opposite result is obtained upon mitogenic stimulation. Zou et al. (1999b) detected increased amounts of IL-2 upon the stimulation of peripheral blood cells with *Staph. aureus.* Moreover, Nitta et al. (1994) demonstrated that glioma cells themselves secreted IL-10 thereby contributing to cytokine

dysregulation in glioma. Another influence on immunosuppression is the secretion by tumour infiltrating lymphocytes of Th2-type cytokines (Roussel et al. 1996).

There are some cytokines that have been shown to regulate both the cell cycle pathway and the growth factor regulated signalling pathway. Van Meir et al. (1990) showed that IL-6 is secreted by glioma cell lines as well as increased in patient's plasma and that it corresponds to the histopathological grade. IL-6 is implicated in the suppression of B-cell maturation which contributes to the production of increased amounts of immune complexes in patient's sera that are associated with a poor prognosis. There are also studies suggesting that IL-6 is implicated in the pathogenesis of astrocytomas. Weissenberger et al. (2004) demonstrated a mitogenicity in IL-6/IL-6Ra transgenic mice while Sayah et al. (1999) showed that complement stimulated astrocytoma tumour cells produced an increase in IL-6 mRNA levels.

IL-8 is also implicated in glioma development and its production is induced by acute phase proteins during the inflammatory response of early tumour development. Desbaillets et al. (1999) suggested that hypoxia in the tumour cell microenvironment causes an increase in the expression of IL-8. IL-8 induces angiogenesis by binding to the chemokine receptors CXCR1 and CXCR2 thereby promoting the invasion of white blood cells and their activation. Samaras et al. (2009) found that the expressions of both IL-6 and IL-8 was increased and that this correlated with increased histological expression of the relevant cytokines. This interesting observation suggested that local tumour expression may be influenced by peripheral lymphocytes able to penetrate the blood-brain barrier.

If either a direct or indirect link from peripheral secretion to tumour cell expression and hence, pathological progress, could be demonstrated, then this would make an analysis and monitoring of peripheral blood cytokine and angiogenic factor secretion an ideal candidate for a clinically useful biomarker.

1.9.5 Use of cytokines in immunotherapy

With the disappointing results of conventional treatment for malignant gliomas, the use of cellular immunotherapeutic treatments has attracted a lot of attention. The clinical trials reviewed by Vauleon et al. (2010) involved LAK (lymphokine-activated killer cells), alloreactive T cells and CIK (cytokine-induced killer cells). Other studies demonstrated an ability of NK (Natural Killer) cells, when activated by IL-2 and IL-15, to destroy tumour cells (Castriconi et al. 2009). Similarly, there have been reports of clinical use of cytokine therapy to activate NK cells to beneficial effect (Castriconi, et al. 2009).

1.9.6 Anti-tumour T-cell activity

Most studies of cellular, anti-tumour responses have focused on cytotoxic T cell production and the production of CD4 and CD8 positive T cells (Zhang et al. 2007). Tumour cells are antigenic and they can be eradicated by autologous T cells (Aoki et al. 2007). In addition tumour cells are able to generate, through the mechanism of specific genetic alterations, many tumour-specific antigens to which a cytotoxic antibody can be raised (Roth et al. 2007). Usually, the immunologically privileged status of the brain is caused, amongst other things, by the endothelial tight junctions. This is given as the reason why high grade brain tumours are able to evade destruction by the host immune defence. However this view has been challenged by the

demonstration by Grigoriadis et al. (2005) who have shown that systemic immunisation with brain-specific antigens can induce an immune response. This immune response is somewhat counteracted by the selective nature of the blood-brain barrier only allowing activated T cells entry to the brain, many of which are immunosuppressive regulatory T cells (Tregs). In addition, they can contribute to the immunosuppressive microenvironment of the tumour caused by high levels of TGF-β. This cytokine has been found to down-regulate the production of IL-1 and HLA (human leukocyte antigen) class II molecules by antigen presenting cells leading to a decreased immune function (Janicki et al. 2008). Other cytokines such as IFN-γ have been shown to facilitate an increase in expression of both HLA class I and class II cell surface molecules of tumour cells. However, most brain tumour patients are chronically immunosuppressed with poor T cell receptor signalling, poor APC function as well as the tumour cells being bathed by a cocktail of immunosuppressive cytokines and Treg cells. Hussain et al. (2006) found that infiltrating macrophages has the cell surface HLA molecules but did not have the usual co-stimulatory CD80, CD86 and CD40 molecules that are required for T cell activation.

1.9.7 Immune response to glioblastoma (GBM)

The process of immunosurveillance in cancer involves the identification and destruction of nascent tumour cells by the immune system (Vesely, et al. 2011). Mouse models have demonstrated that innate and adaptive immune cells, effector molecules and pathways contribute to an extrinsic tumour suppressor mechanism and the increased risk of cancer development amongst immunosuppressed and immunodeficient individuals suggesting a

role for the immune system in cancer avoidance (Vesely, et al. 2011). There are, however, a population of immunocompetent individuals that still get cancer. Dunn, et al. (2012) suggested that an immune system modification must have occurred to generate a tumour promoting role for the immune system. This dual role of tumour promotion and tumour protection is termed immnuoediting and has three phases. These include elimination, which is the recognition of cancer cells by the immune system, equilibrium, in which the expansion of cancer cells is controlled by the immune system and escape, development of immunosuppressive tumour microenvironment, (Dunn, et al. 2012).

The brain has long been thought of as an immunologically privileged site with the tight junctions of endothelial cells providing a blood-brain barrier that prevented the infiltration of T lymphocytes into the CNS. However under the pathological state of a tumour the blood-brain barrier is disrupted and lymphocyte trafficking to the CNS increases (Goldmann, et al. 2006). Previous studies have also demonstrated a long-term resident population of CD8 positive cytotoxic T cells that persist in the brain tissue following infection. They are able to avoid apoptosis and do not return to the circulation (Wakim, et al. 2012).

CD8 positive cytotoxic T cells provide the best response immunologically against tumour cells and, moreover, they have the ability to recognise antigenic peptides through interaction with antigen-presenting cells such as dendritic cells (Guermonprez, et al. 2002). This anti-tumour effect is enhanced by the induction of T lymphocyte production by the stimulatory cytokines IL-2 and IFN-γ secreted by CD4 positive T lymphocytes (Guermonprez, et al.

2002). Microglia are sessile macrophages that respond to signals caused by inflammation and the presence of foreign pathogens (Stoll, 1999) and are implicated in the promotion of glioma through the stimulation of angiogenesis and tumourigenesis (Brandenburg, et al. 2016). They have also been shown to have a role in immunosuppression through secretion of immunosuppressive cytokines TGF-β and IL-10 and the Fas ligand (Badie, et al. 2001). The Fas pathway has been shown to play a role in immune evasion and the Fas ligand is also produced by a sub-population of T lymphocytes, the natural killer (NKT) cells (Tang, et al. 2014).

The brain tumour microenvironment is characterised by an infiltrate of microglia, dendritic cells, macrophages and natural killer cells as well as T lymphocytes. These cells are all implicated in tumour destruction through several different mechanisms of immunosuppression (Watters, Schartner et al. 2005).

1.9.8 Mechanisms of immunosuppression

Tregs are CD4 positive T cells that express α subunit interleukin-2 receptor (CD 25) and the transcription factor FoxP3 (Yomogida, et al. 2013). Previous studies have demonstrated that Tregs infiltrate tumour tissue by creating an immunosuppressant microenvironment and contribute to tumourigenesis and pathogenesis (Heimberger, et al. 2008). In the tumour microenvironment, Tregs influence the downregulation of both CD4+ and CD8+ through different mechanisms such as inducing an immunosuppressive phenotype by interaction with dendritic cells and secreting IL-10 and TGF-β blocking the effector T cell response (Zou, et al. 2010).

Tumour-associated macrophages (TAMs) are the predominant infiltrating leukocyte in most tumours and evidence suggests that they are implicated in tumor initiation, growth, and development (Quatromoni and Eruslanov 2012). There are different phenotypes of macrophage with tumour suppressing (M1) and tumour promoting (M2) activities from differential cytokine secretion. The M2 phenotype mechanism of immunosuppression involves the release of metalloproteases such as membrane type 1-matrix metalloprotease (MT1-MMP) which has been shown to have lytic activity on cellular binding enabling glioma cells to invade the brain parenchyma (Markovic, et al. 2009).

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of cells produced in conditions that involve an altered haemopoietic mechanism such as inflammation and malignancy having a strong immunosuppressive function through interaction with T cells (Raychaudhuri, et al. 2015). These cells utilise several mechanisms in the suppression of T cell function including the secretion of arginase I and indolamime 2,3-dioxygenase (IDO) which act to catabolise the essential amino acids arginine and tryptophan respectively. The also produce nitric oxide (NO) and immunosuppressive cytokines such as TGF-β which activates epithelial mesenchymal transition therefore stimulating migration of malignant glioma neoplastic cells (Mazzoni, et al. 2002). There is also an association between MDSCs and CD4+ effector memory T-cells with the cell surface immune checkpoint receptor programmed death -1 (PD-1). This receptor is expressed on the surface of CD4+ cells and the PD-1L ligand is upregulated on suppressive tumour-derived MDSC (Dubinski, et al. 2016).

The secretion of immunosuppressive factors such as IL-6 and CSF-1 by glioma cells contributes to the Th2 response increasing its activity and

reducing the strength of the anti-tumour effect (Hao, Parney et al. 2002). Other molecules such as prostanoids, IL-10 and the COX-2 enzyme also contribute to the immunosuppressive tumour microenvironment (Gomez and Kruse 2006). These factors combine to facilitate angiogenesis and to induce immunosuppression through stimulating an increase in Tregs, the suppression of dendritic cells, natural killer cells and cytotoxic T cells (Han, et al. 2015). The chemokine CCL-2 is also secreted by glioma cells and is involved in Treg recruitment and migration (Jordan, et al. 2008).

Hypoxia-induced angiogenesis is one of the key models for the cause of microvasculature with GBM. Tumour cells that outgrow endogenous blood supply undergo hypoxia, mediated by HIF-1α transcription. In turn this leads to the transcription of angiogenic factors, vascular endothelial growth factor (VEGF-A), Platelet-derived growth factor ligand BB (PDGF-BB) and follistatin (Charles, et al. 2012a). Hypoxia stimulates the STAT3 pathway which has been shown to play in immunosuppressive role in glioma (Li Hong, et al. 2016).

1.10 Summary of literature review and scope of the study

It is now possible to characterise and evaluate the cytokine and angiogenesis-associated protein secretion from the serum and CSF of patients with malignant glioma with regards to their potential use as a biomarker and in developing novel treatment options. It is now known that cytokine and growth factor expression is a major factor in immune suppression, (Arrieta et al. 2002) and enhancement, but the pattern of expression in blood and brain tissue has never been studied to such a detailed extent before. This study

aims to identify potential blood markers to predict tumour recurrence following surgery that will be quicker and easier to perform than the current method of medical imaging. This will be of great utility in the diagnosis of brain tumours as well as in the grading of tumours. Other aims of this project are to investigate the immunosuppressive properties of white blood cells and to detect the source of a generalised immunosuppression in glioma patient.

1.10.1 Working hypothesis and main aim:

The main aim of this study was to investigate the lymphocytes and glial cells of glioblastoma multiformae patients to determine if they have an altered secretion of cytokines and angiogenesis associated proteins compared with normal patients. It is hypothesised that these can be used as prognostic indicators as well as providing information leading to novel treatment strategies. This altered secretion can be used to detect differences in glioma grade and to enable the clinician to predict tumour progression from low grade to high grade. This may impact upon treatment strategies and allow a glioma screening programme to be developed.

1.10.2 Specific aims

To perform studies in these following areas;

Study 1 – Optimisation of techniques

- To validate and evaluate the bioplex analyser and cell washer for measuring secreted cytokines and angiogenesis-associated factors in a variety of media.
- To perform a lymphocyte viability study on the effects of cryopreservation on cytokine secretion to see if cryopreservation of blood samples is an option for the longitudinal study. This will be done by comparing cell viability measured

by the cell count as well as an analysis of a range of proteins associated with apoptosis.

- To establish the effect of anaesthetic received by patients on the studied analytes

Study 2 – Initial investigations (biomarkers)

- To measure cytokine and angiogenesis associated proteins in GBM patient's plasma before and after resection to see if there is a significant difference in levels.
- To compare serum levels of 8 cytokine and 9 angiogenesis-associated analytes between GBM and normal control patients.
- To perform immunohistochemical analysis of glioma and non-cancerous tissue sections using antibodies raised against elevated serum analytes.

Study 2 – Initial investigations (immunosuppression)

- To perform immunohistochemical staining on sections from GBM and normal control patients to confirm the presence or absence of IL-2 receptors on infiltrating lymphocytes before isolating CD25 positive lymphocytes from the plasma for culture and measurement of cytokine secretion.
- To compare the cytokine and angiogenesis associated protein secretion of peripheral regulatory T-lymphocytes in glioblastoma patients with normal patients.

Study 3 – Experimental investigations

- To compare a wider range of analytes between the sera of different grades of glioma and normal controls.

- To compare a wider range of analytes between the CSF of glioma patients with normal controls.
- To develop a working diagnostic model to distinguish between cancerous and non-cancerous sera and CSF and to distinguish between WHO low and high grade glioma.

Chapter 2

Materials and Methods

For the following the experiments the patient cohorts used are summarised in table 2.1

Table 2.1 Study cohorts, samples and demographics

Study	Patient cohort(s)	Age/gender	Samples	Number
Lymphocyte viability study	high grade glioma (HGG) patients	4 female/ 7 male	Whole blood	11
TNF-α ELISA	high grade glioma patients	4 female/ 7 male	Whole blood	11
Presence of Tregs	high grade glioma patients / menningioma (MGG) patients	HGG - 58-85 years 5 male / 3 female MGG - 24-71 years 3 male / 1 female	Buffy coat	8 HGG / 4 MGG
Role of immunosuppressive cytokines	High grade glioma / non-cancerous control (normal)	**Comparison study** HGG - 38-80 years 21 male / 29 female Normal 26-87 years 21 male / 6 female **Pre vs post op study** HGG - 62-88 years 18 male / 18 female Normal 45-75 years 18 male / 18 female	Serum	50 HGG / 27 normal 36 HGG pre and post op 36 normal pre and post op
Immunohistochemical staining of primary GBM biopsies	High grade glioma / Low grade glioma (LGG) / non-cancerous control (normal)	**Follistatin study** HGG - 33-75 years 6 male / 6 female LGG - 23-60 years 3 male / 3 female Normal 26-67 years 3 male / 3 female **Leptin study** HGG - 29-73 years 10 male / 3 female LGG - 20-59 years 2 male / 2 female Normal 55-81 years 2 male / 2 female **Prolactin study** HGG - 43-81 years 11 male / 1 female LGG - 33-58 years 3 male / 3 female Normal 34-74 years 4 male / 2 female **PDGF study** HGG - 41-79 years 8 male / 10 female Normal 39-85 years 4 male / 2 female	Parrafin embedded tissue	**Follistatin study** 12 HGG, 6 LGG, 6 normal **Leptin study** 13 HGG, 4 LGG, 4 normal **Prolactin study** 12 HGG, 6 LGG, 6 normal **PDGF study** 18 HGG, 6 normal
Blood-borne biomarker study	High grade glioma / non-cancerous control (normal)	**Anaesthetic study** HGG - 55-70 years 5 males / 1 female **Comparison study** HGG - 18-87 years 62 male / 53 female LGG - 18-69 years 23 male / 21 female Normal 26-87 years 17 male / 16 female **Pre/Post op study** HGG pre op - 27-81 years 29 male / 30 female HGG post op - 27-81 years 26 male / 24 female Normal pre op 26-87 years 8 male / 6 female Normal post op 26-86 years 11 females / 8 males	Serum	**Anaesthetic study** 6 HGG **Comparison study** 115 HGG, 44 LGG, 33 Normal **Pre/Post op study** 59 HGG, 19 Normal

2.1 Lymphocyte viability study

2.1.1 Patient Samples

11 glioma patient blood samples were collected from Royal Preston Hospital (RPH) in EDTA tubes. The sample was aliquoted into two separate containers. These were stored at 4°C and -20°C. Each sample contained 1.8 ml of blood. A density gradient medium, lymphoprep, was used to isolate the lymphocytes. These samples were stored at -20°C, and a cell viability analysis, using 0.4% (v/v) trypan blue was performed. The sample was pelleted and re-suspended in 10% (v/v) DMSO and RPMI 1640 media and placed in a cooling chamber and stored at -80°C. The cells were thawed in a 37°C water bath and then pelleted and re-suspended in culture media. Again the cellular viability was analysed using 0.4% (v/v) trypan blue. A human tumour necrosis factor alpha (TNF-α) instant ELISA was completed for samples 2-11.

2.1.2 Isolation of lymphocytes

A volume of 500µl of Lymphoprep™ (Axis-Shield) was placed in a 1.5 ml microfuge tube, and 1000 µl of the blood sample was carefully placed on the top of the density gradient medium. The sample was centrifuged at 800 x g for 20 minutes. A lymphocyte layer was formed between the blood sample and the medium which was removed. These cells were decanted into two 1.5 ml microfuge tubes. The first tube had an equal amount of 0.4% (v/v) trypan blue. 50 ul sample of this sample was used for cell counting using a haemocytometer. In the second tube 1 ml of PBS was added and 650 µl was taken into another microfuge tube for cryopreservation. The rest of the sample

was centrifuged at 3000 x g for 1 minute and the supernatant collected and stored at -20°C. This sample was used for the ELISA.

2.1.3 Cryopreservation of Lymphocytes

650 µl of lymphocyte and PBS solution was centrifuged at 3000 x g for 1 minute and the supernatant discarded. The remaining pellet was then re-suspended and stored. RPMI 1640 culture media containing 10% (v/v) foetal bovine serum was mixed with 10% (v/v) dimethyl sulphoxide (DMSO). 1.0 ml of the culture media/DMSO solution was mixed with the lymphocyte/PBS solution and the sample was pelleted and placed in a cooling chamber and stored at -80°C. The cooling chamber mediated a cooling rate of 1°C/min. The samples were then thawed using a 37°C water bath for 7 minutes and centrifuged for 1 minute at 2400 x g. The supernatant was discarded, and the pellet was re-suspended in 1.0 ml RPMI 1640.

2.1.4 Human TNF-α ELISA

A human TNF-α instant ELISA (ebioscience) kit was used to measure the concentration of TNF-α in the paient samples. Columns A-G, rows 1-2 of the microtitre plate were used for reference standards with particular amounts of TNF-α, (table 3.4). The kit was stored at -20°C and removed from the freezer on the day of use. A volume of 100 µl of distilled water was added to each well in the microtitre plate including the standard wells. 50 µl of sample from the patient samples was added to the wells. The microtitre plate was covered and the samples were mixed using a microplate shaker. The plate was then incubated for 3 hours at 22°C.

The plate cover was removed and the wells were washed 6 times with 400 µl wash buffer (PBS pH7.0 plus surfactant). Between each wash excess buffer was removed by tapping the plate on tissue paper. Whilst the plate was still wet, 100µl of tetramethyl-benzidine substrate solution was added to all wells. The plate was incubated for 10 minutes at 22°C. The enzyme reaction was stopped after 10 minutes by adding 100µl of 1.0M phosphoric acid stop solution into each well. A Bio-tek plate-reader was used to measure the absorbance of the wells. This was read at a wavelength of 450 nm.

2.2 Presence of Tregs in high and low grade gliomas

2.2.1 Sample Collection

Buffy coat samples were used in this study. This is the middle fraction of centrifuged blood that contains platelets and white blood cells. They are prepared by centrifuging the blood sample at 200 x g for 10 minutes with the centrifuge brake off. The white layer that sits above the red blood cell fraction is the buffy coat and is carefully removed. The buffy coat samples were from the Royal Preston Hospital and consisted of glioblastoma multiforme (GBM) (Grade IV) and Meningioma (Grade I) patients. These were collected within ethical guidance and clearance from the Brain Tumour North West (BTNW) tissue bank. Patient demographics used for the study were taken, (see Table 2.1), and the age ranges for the two groups were GBM, (58-85 years) and meningioma, (24-71 years). The volume of the buffy coat samples received was variable and ranged from 0.1ml to 1.5 ml. The samples were stored in a -80°C freezer and thawed slowly at 4°C before use.

An Horiba ABX Pentra 60 C + haematology analyser was used to provide the total WBC (White Blood Count) which was used as the absolute cell number in the buffy coat sample. The WBC count was given as x 10^3 cells/mm^3 . This was diluted to 1x10^6 cells/ml which is the required concentration according to the kit manufacturer's instructions. The volume of samples required for this dilution was calculated thusly:

$$\text{Sample volume of } 1\text{x } 10^6 \text{ cells per ml} = \frac{\text{(Total volume of sample)}}{\text{WBC}}$$

2.2.2 Cell thawing and Cell Viability

Trypan blue was used to measure cell viability and the cells were counted using a haemocytometer. There were two methods of thawing of the sample taken from the -80°C freezer. Slow thawing was done using a 4°C refrigerator and fast thawing using a 37°C water bath.

2.2.3 Treg Detection

The buffy coat samples were used in the analysis of the Treg compartment using Miltenyi Biotec 'Treg Detection Kit (CD4/CD25/FoxP3) (PE), human'. This kit comprised of surface staining antibodies: anti-CD4 (VIT4)-FITC, and anti-CD25-APC and the intracellular staining antibody: Anti-FoxP3-PE, FcR blocking reagent, fixation/permeabilization solution I, fixation/permeabilization Solution II, and 10X permeabilization buffer. The buffer used for the analysis was a solution of phosphate-buffered saline (pH 7.2), 0.5% (v/v) bovine serum albumin and 2mM Ethylene diamine tetra acetic acid (EDTA).

The labelling procedure used was that provided by the manufacturer. The cell suspension was centrifuged at 300 x g for 10 minutes. The supernatant was

aspirated and $1x10^6$ cells were re-suspended. 10 µl of anti-CD4-FITC and 10 µl of anti-CD25-APC antibodies were added to the cell suspension and this was then incubated for 10 minutes at 4°C in the dark. The cells were washed with 1.0 ml of buffer and centrifuged at 300 x g for 5 minutes at 4°C. To perform the intracellular labelling cells were incubated with 1ml of a 1:4 dilution of fixation/permeabilization solution I and fixation/permeabilization solution II. This was done for 30 minutes at 4 °C in the dark. The cells were washed with 1X permeabilization buffer and FcR blocking reagent was used to prevent non-specific binding. 10 µl of Anti-FoxP3-PE were added to the suspension for 30 minutes at 4 °C in the dark. The cells were then washed and centrifuged at 300 x g and the remaining cell pellet was re-suspended in 400 µl buffer.

2.2.4 Analysis of Tregs using flow cytometry

The labelled samples were analysed using the Guava® easyCyte HT sampling flow cytometer. 200µl of each sample was added to the 96 well plate. Table 2.2 describes the excitation and emission wavelengths used for the set up of the flow cytometer.

Table 2.2. Table showing flow cytometry setup.

Antibody	Fluorophores	Excitation wavelength (nm)	Emission wavelength (nm)	Filter (max/BP)	Laser (Fluorescence)
CD4	FITC (fluorescein isothiocyanate)	488	519	530/30	Green- Blue
CD25	APC (Allophycocyanin)	633	660	660/20	Red- Red
Anti-FoxP3	PE (Phycoerythtin)	488	578	575/26	Yellow- Blue

2.3 Bioplex method/optimisation

A luminex suspension array system (Biorad) was used to quantify the cytokine concentrations.

The Bio-Plex system permited multiplexing of theoretically up to 100 different ELISA-type assays within a single sample. Each assay was performed on the surface of a 5.5 μm polystyrene bead. The beads were filled with different ratios of two different fluorescent dyes, resulting in an array of 100 distinct spectral addresses. Each set of beads could be conjugated with a different capture molecule; the conjugated beads can then be mixed and incubated with the sample in a microplate well to react with specific analytes. A fluorescently labelled reporter molecule that specifically binds to the capture molecule is added. Precision fluidics align the beads in a single file through a flow cell where two lasers excite the beads individually. The red classification laser excited the dyes in each bead, thereby identifying the specific bead address. The green reporter laser excites the reporter molecule associated with the bead, allowing quantitation of the captured analyte.

A verification and calibration procedure of the luminex analyser was carried out according to the manufacturer's instructions before analysis took place. The manufacturer supplied reagents from different kit batches which were used to confirm the accuracy, precision and reproducibility of the analytical technique used. A range of samples were tested using reagents from different batches and the results compared. Samples were performed in duplicate on the 96 well plate alongside the standard curve used to generate the results. Two wells with only manufacturer's diluents were used as blanks and the

resultant background reading levels were subtracted from all sample results. One sample from a single patient was included on each plate to control for inter-plate reproducibility.

2.3.1 Role of immunosuppressive cytokines

Serum samples from 50 GBM (grade 4 glioma) patients were analysed along with 27 non-cancerous control patients. Sera were also analysed from 36 patients with GBM who underwent biopsy or debulking of tumour. The sera were collected both immediately pre-operatively and within 24 hours of surgery.

Control serum samples were also collected immediately pre and post-operatively within 24 hours of surgery from 36 patients with no known history of malignancy. These patients underwent elective surgery for a non-cancerous pathology.

The levels of 8 cytokines and 9 angiogenesis-associated factors were simultaneously evaluated using the Bioplex analyser supplied by BioRad.

The cytokines measured were IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF-γ and TNF-α.

The angiogenesis-associated factors were angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, PECAM-1, VEGF.

2.4 Immunohistochemical staining of primary GBM biopsies

Formalin-fixed, paraffin embedded tissue sections were obtained with ethical clearance from the Brain Tumour North West (BTNW) tissue bank at the University of Central Lancashire.

Slides were deparaffinised using two Sigma histoclear rinses followed by two 90% ethanol rinses. Antigen retrieval was performed in citrate (pH 6) buffer in the pre-treatment (PT) link machine (Thermo Shandon) set at 97 °C for 40 minutes. Residual endogenous peroxidase activity was quenched using a 0.3% (v/v) hydrogen peroxide solution for 15 minutes. The staining procedure followed the manufacturer's instructions using the Vectastain Elite ABC Kit: PK-7200. PBS pH 7.4 was used for the washing reagent as well as for antibody dilution. Horseradish peroxidase (HRP) enzyme and 3,3′-diaminobenzidine (DAB) substrate was used to localise peroxidase activity. DAB was made up to manufacturer's instructions. The slides were counterstained using haematoxylin for 1 minute and washed with hot tap water. The slides were then dehydrated using two methylated spirit and two Sigma histoclear rinses. The slide were then mounted for inspection by at least two investigators. Immunohistochemical staining was performed using the antibodies and dilutions in table 2.3. The optimum dilutions were determined by preliminary experimental staining.

Table 2.3 The antibodies used in the immunohistochemistry of glioma and non-cancerous tissue sections.

Antibody	Dilution	Species/clonality	Supplier
Follistatin	1:250	Rabbit/ polyclonal	Abcam
Leptin	1:500	Rabbit/ polyclonal	Abcam
Prolactin	1:500	Rabbit/ polyclonal	Abcam
PDGF-AA	1:200	Rabbit/ polyclonal	Abcam
PDGF-BB	1:200	Rabbit/ polyclonal	Sigma
PDGFRα	1:200	Rabbit/ polyclonal	Sigma
PDGFRβ	1:200	Rabbit/ polyclonal	Sigma

A scoring system was applied to analyse the intensity of staining of the tissue sections (Table 2.4).

Table 2.4. Scoring system for stained sections

0	No staining
1	Weak staining
2	Moderate/intermediate staining
3	Strong/intense staining

2.5 Enzyme-linked Immunoassay (ELISA)

The samples for this study were obtained with ethical clearance from the Brain Tumour North West (BTNW) tissue bank at the University of Central Lancashire.

The serum serum was stored at -80 °C and thawed at 22°C.

The ELISA kits used were from Sigma Aldrich for the PDGF-BB (RAB0397) and PDGF- AA (RAB0394) ligands. A volume of 200 µl of each standard was added to the first column on an antibody coated ELISA plate. A serial dilution of the standard solution was made. This dilution covered 400pg/ml – 0pg for PDGF-BB and 30,000pg/ml – 0pg/ml for PDGF-BB. A volume of 100 µl of each serum sample was added to specific wells of the ELISA microtitre plate. Each sample was added in duplicate. Control wells were made using all of the reagents with the omission of the patient samples. The wells were covered and the plate was gently agitated overnight at 4°C. The wells were emptied of liquid and washed four times with wash buffer (PBS plus surfactant). The liquid was decanted after the last wash. The detection antibody was prepared and added to the wells of the microtitre plate. The plate was incubated for 1 hour at 22°C with gentle agitation. The liquid was discarded and the wells were washed using wash buffer. HRP-Streptavidin was added to each well and the microtitre plate was incubated for 45 minutes at 22°C with gentle agitation. The liquid was discarded and the wells washed using wash buffer. A volume of 100 µl Colorimetric TMB reagent was added to each well and incubated at 22°C in the dark with gentle agitation. A volume of 50 µl of stop solution was added to each well and a colourimetric reaction took place within the wells. The microtitre plate was then read spectroscopically at 450nm in an

Omega 96 well plate reader. The mean absorbance for the duplicate standards and samples was then calculated. The absorbance from the control wells was subtracted from the sample wells. Myassay.com was used to plot the standard curves in order to determine the serum concentrations of the analysed molecules.

2.6 Blood-borne biomarker study

2.6.1 Tissue/serum collection

Serum samples were collected from 115 patients with glioblastoma (WHO Grade IV) and 44 patients with 'low grade (WHO Grade II) of which 25 were astrocytomas (grade II), 15 were oligodendrogliomas (grade II) and 4 were ependymomas (grade II) using the Louis 2007 WHO classification system. A total of 33 samples from patients with no history of cancer were taken from pre-operative clinics and from volunteers with informed consent. Samples were obtained from the Walton Research Tissue Bank and the Brain Tumour North West tissue bank under ethical clearance no. 09/H0304/88. UCLan's School of Pharmacy and Biomedical Science's Health and safety committee also gave ethical approval on 22nd February 2010. Only newly diagnosed patients with a histopathological diagnosis of WHO Grade II and IV were included. The glioblastoma patients were all given a high dose, (16mg/day), of Dexamethasone. A total of 23 of the high grade glioma patients were treated with Temozolomide. The low grade glioma patients and the non-cancerous controls were not on any oncological medication. Informed consent was taken pre surgery by consultant neurosurgeons. All pre-operative high grade blood samples were taken in theatre whilst the patient was under anaesthetic and

low grade blood samples were taken in the outpatients clinic. Post-operative samples were taken within 24 hours of debulking. The serum tubes were left to clot at room temperature for a minimum of 30 minutes and a maximum of 2 hours from blood draw to centrifugation. Separation of the clot was accomplished by centrifugation at 1,200 x g for 10 minutes and 500 µl aliquots of serum dispensed into prelabelled cryovials. Serum samples were snap frozen using liquid nitrogen and stored at -80^{o}C.

Cerebro-Spinal Fluid (CSF) samples from 8 glioblastoma patients and 8 non cancerous patients were taken by Lumbar puncture at Athens Hospital**.** The samples were taken using a local anaesthetic prilocaine and were taken prior to surgery. The patients were not on any other medication.

2.6.2 Clinical data collection

The 115 glioblastoma patients comprised of 42 females (mean age 57.8 years) and 73 males (mean age 61.5 years). The 44 low grade patients comprised of 21 females (mean age 48.6 years years) and 23 males (mean age 48.4 years). The 33 control samples comprised of 13 females (mean age 58.1 years) and 20 males (mean age 61.7 years). Survival data from 53 glioblastoma patients was analysed for correlation with the serum levels of all significantly raised biomarkers.

2.7 Statistical analysis

2.7.1 Statistical analysis of Treg study data

All measurements were performed in duplicate and statistical analysis was performed in Microsoft Excel. The results were analysed using a two-tailed paired samples t-test (cell viability) and a two-tailed pearson’s correlation

coefficient (ELISA). The mean, standard deviation and standard error of the relative amount of CD4+CD25+FoxP3+ Tregs was measured for both GBM and meningioma groups. The differences in mean were shown using an histogram plot with error bars delineating the standard error. A student's t-test (two-sample assuming unequal variances) was performed to compare the patient groups for both the analysis of Treg and serum and CSF cytokine levels. The statistical tests were carried out using a $p<0.05$ significance level.

2.7.2 Statistical analysis of blood-borne biomarker data

Significance between groups was measured using a paired t-test and correlations (study 1) and a Mann-Whitney U test assuming non-Gaussian distribution (studies 2, 3 and 4). The survival data was examined for correlation with each anylate level using the CORREL function on Microsoft Excel. A $P<0.05$ was considered statistically significant. Power analysis was performed using an online calculator (powerandsamplesize.com) on the significant potential biomarkers in study 2 to determine the minimum sample size required for 80% power using a difference of 2 standard deviations to distinguish between glioma and non-cancerous sera and between low and high grade glioma.

Chapter 3

Results

3.1 Lymphocyte viability study

The rationale for this part of the study was to determine the effect of cryopreservation on lymphocytes, to determine if the method of storage had an effect upon cytokine secretion.

3.1.1 Cell Viability

Lymphocyte cell viability results for the 4°C stored samples are shown in Table 3.1.

Table 3.1. The percentage viability for lymphocytes stored at 4°C using trypan blue.

Patient	Sample No	Viable	Non-Viable	Age of sample *	% Viability
0	897	204	18	6	91.89
2	900	127	13	8	90.71
3	901	668	10	7	98.52
4	903	88	5	7	94.62
5	904	302	74	5	80.32
6	905	389	44	5	89.83
7	906	398	35	4	91.91
8	910	178	15	5	92.22
9	911	374	32	6	92.12
10	912	443	12	6	97.36
11	914	701	63	13	91.75

* The age of the sample is given as the number of days from collection from Royal Preston Hospital to testing

The sample from patient 1 was severely haemolysed therefore there was no result for this sample. In this study the mean viability was 91.93% and the standard deviation was 5.46.

Lymphocyte cell viability results for the -20°C stored samples are shown in (Table 3.2).

Table 3.2. The percentage viability for lymphocytes stored at -20°C using trypan blue.

Patient	Sample No	Viable	Non-	%
0	897	78	6	99.3
2	900	66	12	84.6
3	901	22	112	16.4

In this study the poor results for patients 2 and 3 demonstrated that the -20°C storage destroyed the integrity of the cells. The cryopreservation and freezing protocol was performed for the rest of the study samples. Lymphocyte cell viability results for the -80°C stored samples are shown in Table 3.3.

Table 3.3. The percentage viability for lymphocytes cryopreserved and stored at -80°C using trypan blue.

Patient	Sample No	Viable Cells	Non-Viable Cells	% Viability
4	903	19	60	24.05
5	904	13	32	28.9
6	905	8	33	19.51
7	906	16	9	64
8	910	725	3015	19.38
9	911	28	65	30.11
10	912	545	5866	8.05

Patient 11 was not included in the study as this sample was a fresh sample and was not cryopreserved. The mean viability was 27.71% and the standard deviation was 16.43.

Overall the results demonstrated that the lymphocyte viability was significantly decreased in samples that had been cryopreserved compared with those that had been stored at 4°C.

3.1.2 ELISA study

Figure 3.1 shows the development of colour in some of the wells of the microtitre plate after 10 minutes of adding the Stop solution.

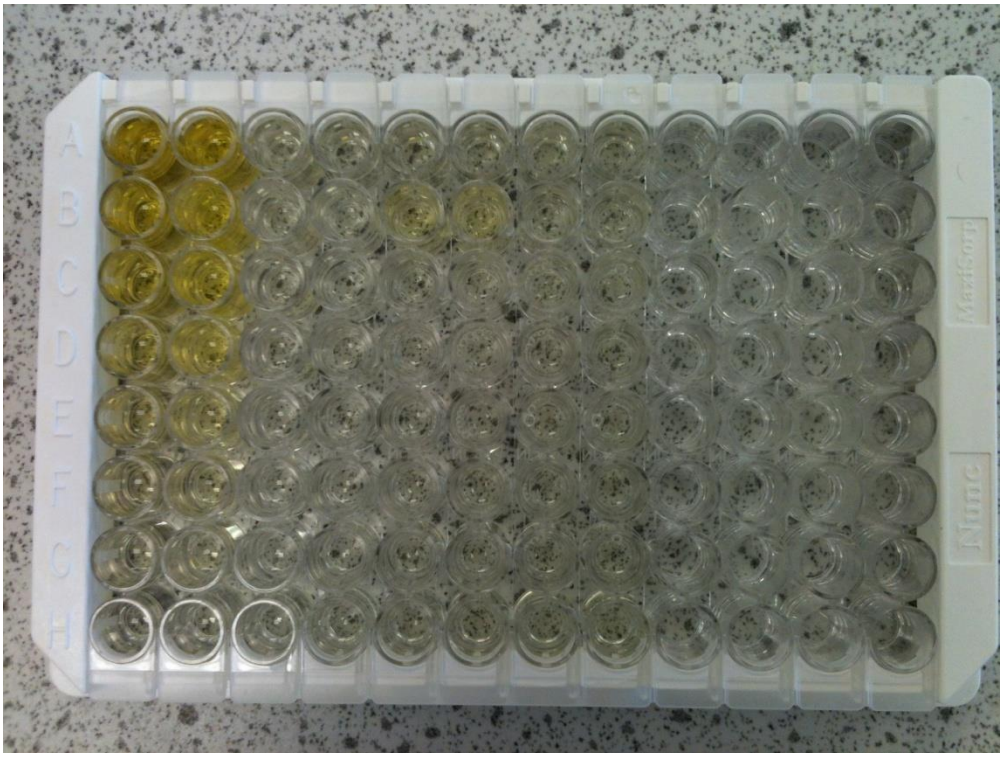


Figure 3.1. An original photograph showing the ELISA microwell plate. The standards are shown in column 1 and 2.

The absorbance readings for the standards in the ELISA was set at 450nm and the data are shown in table 3.4 for information.

Table 3.4. The absorbance of the ELISA standards read at 450 nm.

Concentration of TNF-α pg/ml	Absorbance at 450 nm
500	2.62
250	1.58
125	0.92
62.5	0.49
31.3	0.27
15.6	0.2
7.8	0.15

From the data a standard curve was produced and this is shown in figure 3.2

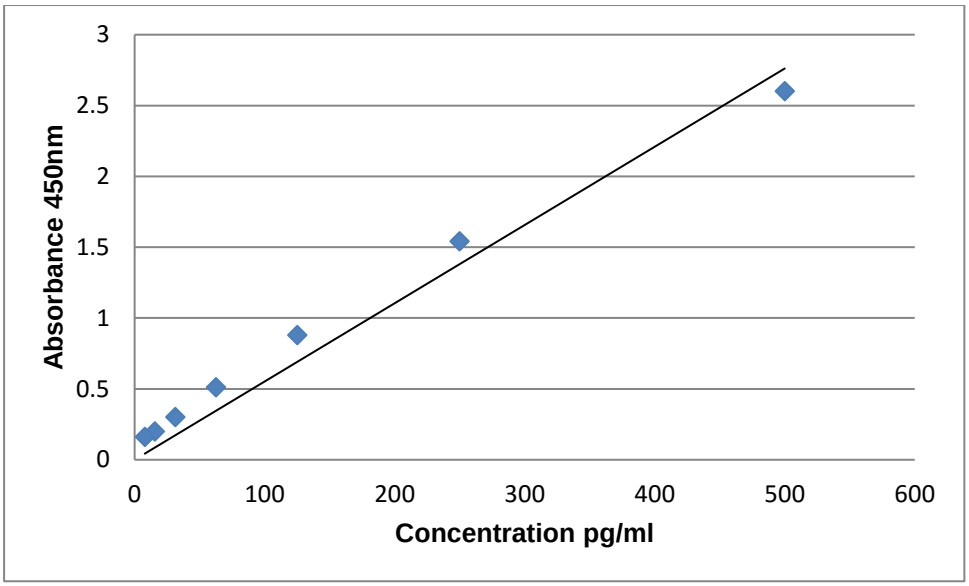


Figure 3.2 The absorbance at 450 nm of standard concentrations of TNF-α.

The TNF- α concentrations were determined using the standard curve and are given in Table 3.5. These concentrations were multiplied by 2 to account for the dilution factor from the ELISA protocol.

Table 3.5. The concentrations of TNF-α in patient samples 2-11. Data are the mean for 2 readings from each patient.

Patient	Mean Absorbance	[TNF-α] / 2 pg/ml	[TNF-α]	Age of
2	0.107	12.6	25.2	8
3	0.098	12.4	24.8	7
4	0.096	12.4	24.8	7
5	0.094	12.4	24.8	5
6	0.088	12.3	24.6	5
7	0.088	12.3	24.6	4
8	0.093	12.4	24.8	5
9	0.094	12.4	24.8	6
10	0.109	12.6	25.2	6
11	0.269	42	84	13

3.1.3 Statistical analysis for cellular viability data.

A two-tailed, paired samples, t-test was performed. This showed a significant difference in cellular viability between the refrigerated and cryopreserved samples when using Trypan Blue with the samples stored in the fridge having a. higher viability than the cryopreserved samples, ($t(7)=5.029$, $p<.01$).Consequently all future samples were refrigerated.

3.1.4 Statistical Analysis for ELISA data

A two-tailed Pearson's correlation coefficient was performed. This shows no significant correlation between the concentrations of TNF-α and the sample age. ($r=0.886$, $n=10$, $p<0.01$). Although this p-value result suggests a positive correlation the experiment was affected by the single outlier of sample 11 and so these results were discounted. With the outlier excluded the significance disappears ($r=0.650$, $n=9$, $p>0.05$). A greater number of samples are required

to verify if this was a rogue result or an indication of increasing production of TNF-α connected to the age of the sample.

There was also no correlation between age of sample and viability using trypan blue, ($r=0.230$, $n=10$, $p=0.523$). There was no correlation between the concentration of TNF-α and the percentage viability using trypan blue, ($r=0.104$, $n=10$, $p=0.776$).

3.2 Presence of Tregs in high and low grade gliomas

Data from the previous study indicated that cryopreservation of lymphocytes was not the optimum method of storage for these cells for studying both the cell surface proteins and the secretions of the preserved cells. The availability of buffy-coat samples collected by the Royal Preston Hospital was an attractive option to collect an adequate number of samples for this study.

Jacobs, et al. (2012) reported an increase in the Treg population in the peripheral blood of malignant melanoma patients compared with patients with benign tumours with increased in the number of Tregs correlated with grade of tumour. This experiment was therefore performed to evaluate whether such a difference was evident in the peripheral blood of high grade (glioblastoma) patients and low grade (meningioma) patients.

The results show that thawing at 4°C resulted in a greater number of viable cells than thawing at 37°C. The average percentage of viable cells for the samples thawed at 4°C was 69.69% and for samples thawed at 37°C was 54.58% as shown in Tables 3.6 and 3.7. As a result, each sample was thawed at 4°C.

Table 3.6. The percentage of viable cells from slow thawing.

Sample no.	Percentage of viable cells
1871	78.2
1891	53.72
1880	28.45
2231	78.9
2242	86.54
2251	73.21
2253	88.79
Average	69.69

Table 3.7 The percentage of viable cells from fast thawing.

Sample no.	Percentage of viable cells
1889	10.1
1892	11.34
1877	18.57
1932	88.34
1937	79.02
2254	93.45
2261	81.23
Average	54.58

3.2.1 Treg Quantification

The WBC count and the volume of the sample were different for each sample. Samples with $1x10^6$ cells/ml or more ($>1x10^6$ cells/ml) were used for the Treg detection method. The total WBC count for each sample is given in Table 3.8.

Table 3.8. The WBC and volume taken of the samples used in the study

Sample no.	WBC count ($x10^6$ cells/ ml)	Total volume of sample (ml)	Volume taken (µl)
GBM Patients (8)			
1893	20.6	0.5	97
2215	15.3	0.5	130
2225	9.5	1.3	210
2238	0.1	1.5	1500
2251	2.5	0.7	400
2209	15.7	1	64
2263	87.4	0.1	11.44
1956	14.2	0.5	70
Meningioma Patients (4)			
1906	11.1	1	90
1910	11.1	0.5	90
1959	19.3	1	103.6
1969	10.2	0.5	196

3.2.2 Analysis of immunosuppressive Tregs using Flow cytometry

Lymphocytes were gated using the forward and side scatter dot plot. The percentage amount of CD4+CD25+FoxP3+ Treg cells was measured using the CD25+FoxP3+ cell population transferred to the CD4+ gate, (see figure 3.3). In this figure A and B are two gatings on samples from the same patient. The left hand boxes are a plot of forward scatter versus side scatter. The cloud in the lower right hand corner with high forward scatter and low side scatter are the lymphocyte population according to the manufacturer's instructions. There is significant cellular debris in this plot. This gate is then used to analyse the CD4+ population which isolates T lymphocytes from the rest of the lymphocyte population. This is shown in the middle two boxes of figure 3.3. Finally this gating is transposed to the third box on the right hand side of figure 3.3 which is a plot of FoxP3 versus CD25. Cells in the upper right quadrant are therefore CD4+CD25+FoxP3+ cells which are the Treg population. Naive T cells (CD4+CD25-) were identified in the same way.

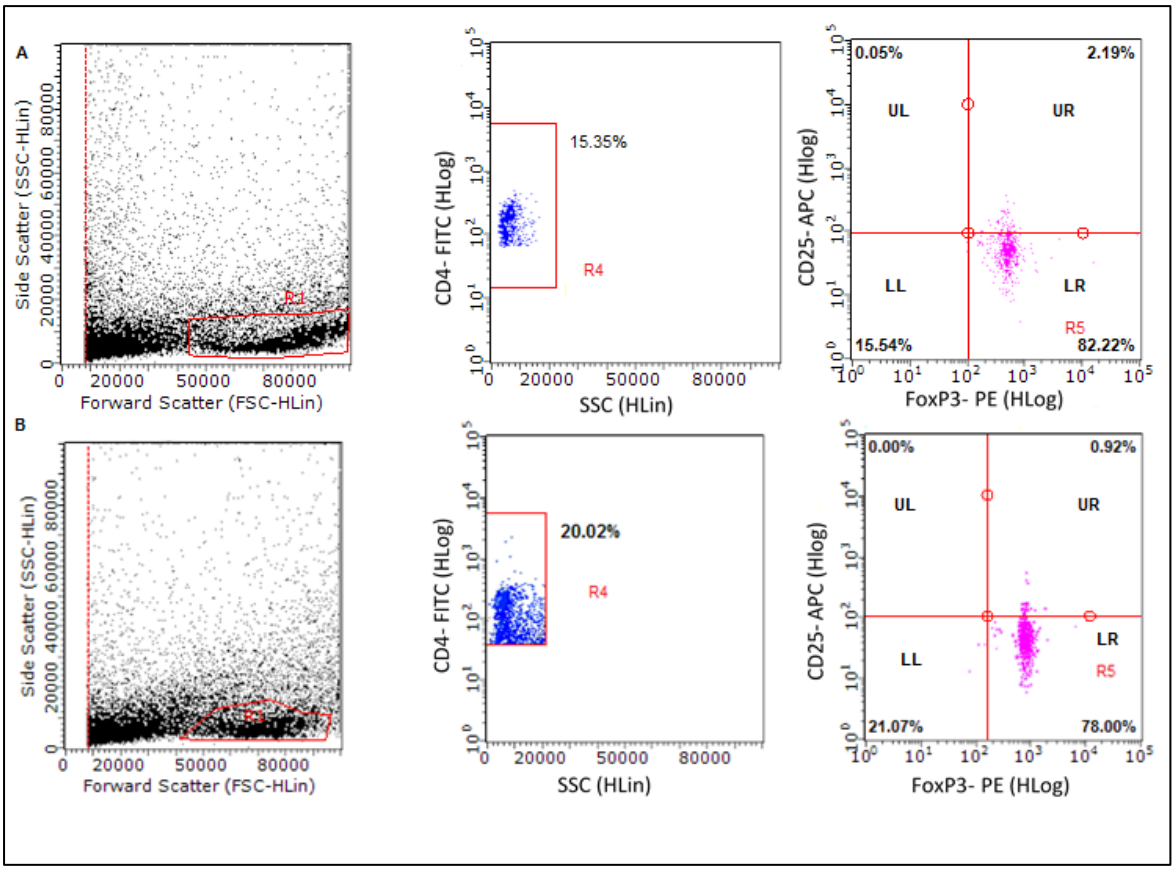


Figure 3.3. Flow cytometry of regulatory T cells. A and B are two gatings from the same patient.

The mean, standard deviation and standard error of the mean of the percentage of CD4+CD25+FoxP3+ cells (Tregs) for both groups is shown in Table 3.9. These results were lower (1.71 ± 0.217%) in GBM patients compared with meningioma patients (2.19 ± 0.45%). The standard deviation was 0.619 in GBM and 0.9 in meningioma patients. The data show no significant difference between the two groups.

Table 3.9. CD4+CD25+FoxP3+ cells (%) in GBM and meningioma buffy coat samples with Mean, Standard Deviation and Standard Error.

Sample	%Tregs in GBM	% Tregs in Meningioma
1	1.82	3.21
2	1.98	2.18
3	0.34	2.34
4	1.47	1.02
5	2.21	
6	2.25	
7	1.67	
8	1.96	
Mean	**1.71**	**2.19**
Std. Dev	**0.612**	**0.9**
Std. error	**0.217**	**0.45**

3.2.3 Statistical analysis

A Student's t-test with unequal variance was performed on the mean T regs amount in both cohorts of patients with p= 0.418 which indicate no significant difference between the groups.

3.2.4 Analysis of naïve Treg cell population

Naïve T cells are CD4+CD25- T cells produced by the bone marrow and have undergone differentiation in the thymus but have not yet encountered an antigen. This analysis was done to determine whether there were significant differences between the two cohorts in this population of cells that were later normalised following conversion of cells to the immunosuppressive Treg subtype.

The results show that there was no significant difference ($p>0.05$) in the amount of naïve (CD4+CD25-) T cells in GBM (10.04%) and Meningioma (11.56%) as shown in table 3.10.

Table 3.10. Data showing CD4+CD25- T cells (%) in GBM and Meningioma patient sample with calculated Mean, Standard Deviation and Standard Error.

Sample	%Tregs in GBM	% Tregs in Meningioma
1	12.34	12.45
2	9.26	9.58
3	28.64	9.32
4	7.02	14.89
5	9.1	
6	8.56	
7	4.93	
8	0.47	
Mean	**10.04**	**11.56**
Std. Dev	**8.3**	**2.63**
Std. error	**3.54**	**1.32**

A Student's t-test with unequal variance was performed on the mean naïve T cell amount in both cohorts of patients with $p= 0.717$ which indicate no significant difference between the groups.

3.3 Role of immunosuppressive cytokines

Data from the previous study indicated that the number of regulatory T cells was not increased in patients with both malignant and non-malignant tumours of the CNS. This work aimed to establish whether there were any differences in circulating serum cytokine levels of these patients compared to non-cancerous control patients and the possible site of production of these cytokines.

These results show that there were significant differences ($p<0.05$) in serum analytes between pre and post-operative levels for three of the eight cytokines IL-6, IL- 8, IL-10 ($p<0.05$) (see figure 3.4). Moreover, there was also a significant difference ($p<0.05$) between pre and post-operative levels for three of the eight cytokines IL-6, IL-8 and IL-10 measured when compared to the control values.

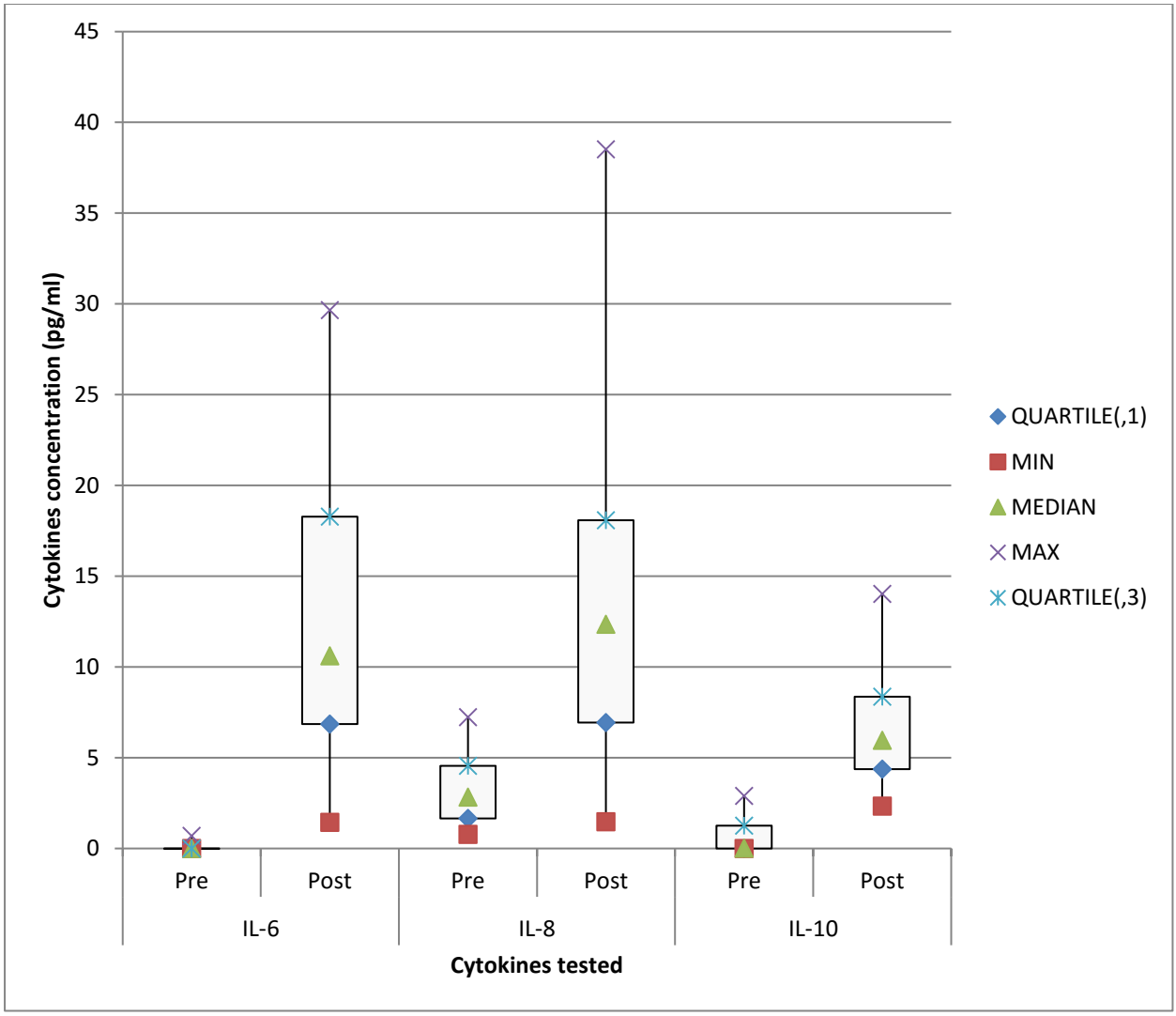


Figure 3.4. The concentrations (pg/ml) of IL-6, IL-8 and IL-10 in the sera of pre and post-operative patients. Data are mean +/- 2SD, $p<0.05$, n = 36 paired samples from GBM patients undergoing surgery for removal of tumour.

The serum analytes that do not show a statistical difference ($p<0.05$) between non-cancerous control serum and GBM are G-CSF, HGF, Leptin and VEGF, (See table 3.12).

The serum analytes show a statistical difference ($p<0.05$) between non-cancerous control serum and GBM are angiopoietin, follistatin, IL-8, PDGF-BB and PECAM-1. (See figure 3.5).

Table 3.11 Mean values and standard deviations of angiogenesis factor concentrations for non-cancerous (n=27) and high grade (n=50) glioma sera samples with no significant difference.

Cytokine		Mean (pg/ml)	standard deviation	n	paired differences	significance p<0.05
G-CSF	non-cancerous	3.1	0.29	27		no
	high grade	3.1	0.61	50	0	no
HGF	non-cancerous	738.9	79.6	27		no
	high grade	724.7	142.3	50	14.2	no
Leptin	non-cancerous	9102.6	1533	27		no
	high grade	10312.3	1685.3	50	1209.7	no
VEGF	non-cancerous	85.2	5.5	27		no
	high grade	75.4	20.9	50	9.8	no

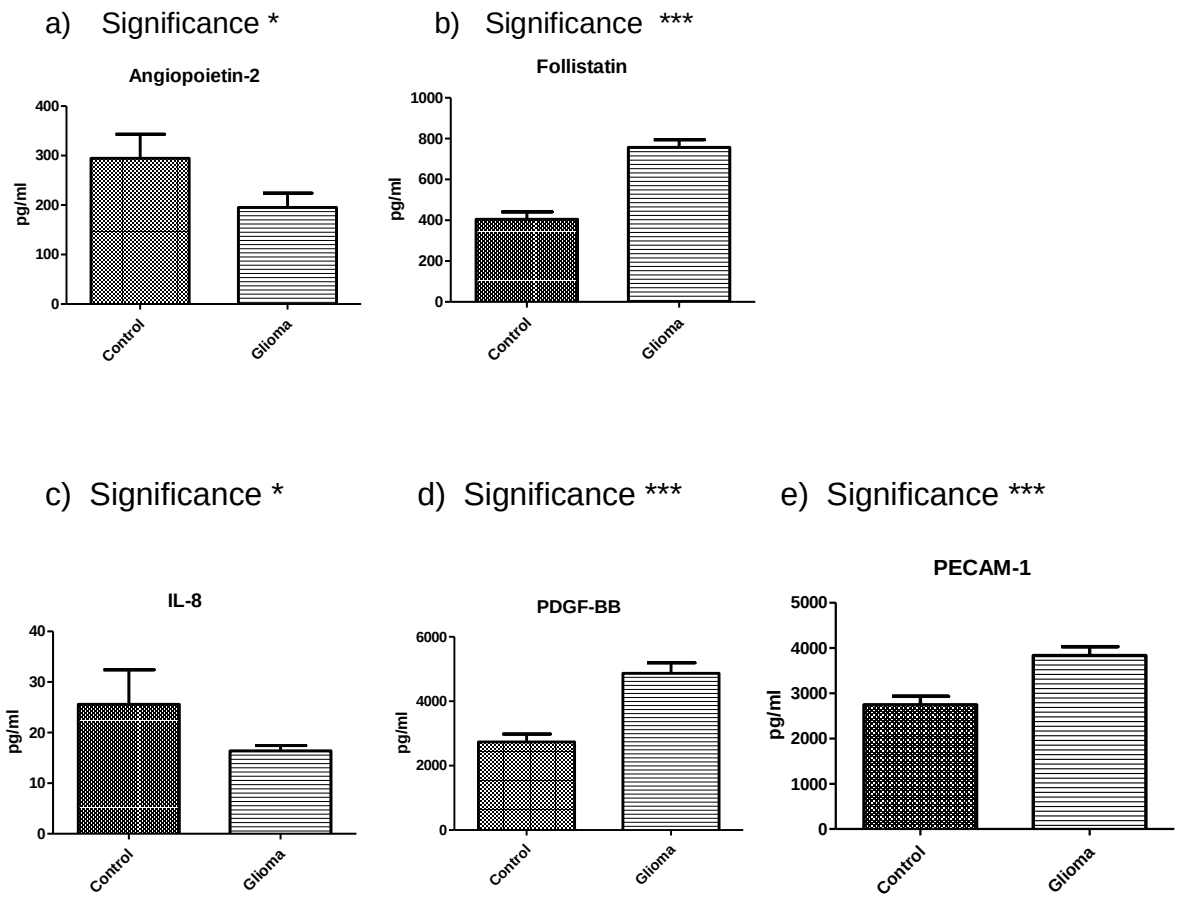


Figure 3.5 **Bar charts a) to e) show the analyte levels (pg/ml) between glioma and non-cancerous serum. Data are mean +/- 2sd, n=50 glioma, 27 non-cancerous control, p<0.05 for glioma compared to non-cancerous control. The significant differences * = p<0.05, *** = p<0.001, between non-cancerous control and high grade glioma sera are shown above the histograms.**

3.4 Immunohistochemistry of follistatin

The aim of this study was to determine the likely source of production, either from the tumour cells or elsewhere, of the increased biomarkers follistatin, to determine if there were any differences in the pattern of expression between low and high grade glioma tissue specimens. The study analysed 12 high grade, 6 low grade and 6 non-cancerous specimens. The stained specimens were graded as 1, 2 or 3 according to the intensity of staining in the nucleus, cytoplasm or interstices using the scoring system outlined in chapter 2.

3.4.1 Results of follistatin staining

High grade, low grade and non-cancerous glioma – Follistatin staining

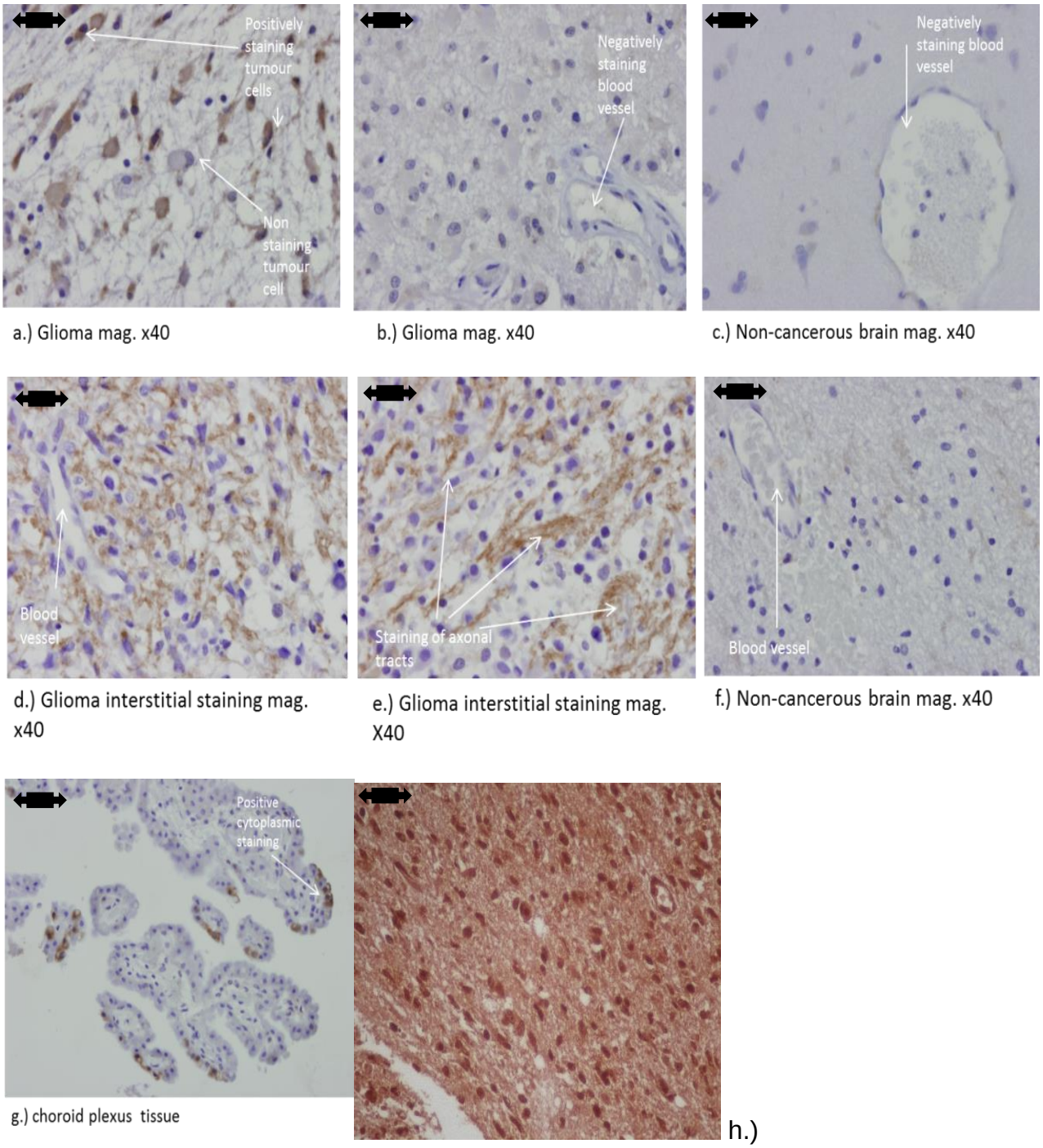


Figure 3.6 **Original photographs showing follistatin staining in both high grade glioma, (a),(b),(d),(e) and non-cancerous brain sections, (c) and (f) mag x40. Typical staining of 12 high grade and 6 non-cancerous sections from different patients. (g) shows choroid plexus tissue staining. (h) shows follistatin staining in a low grade glioma mag x100. Typical staining of 6 sections from different patients. Black scale bar represents 20 microns (a-g) and 100 microns (h)**

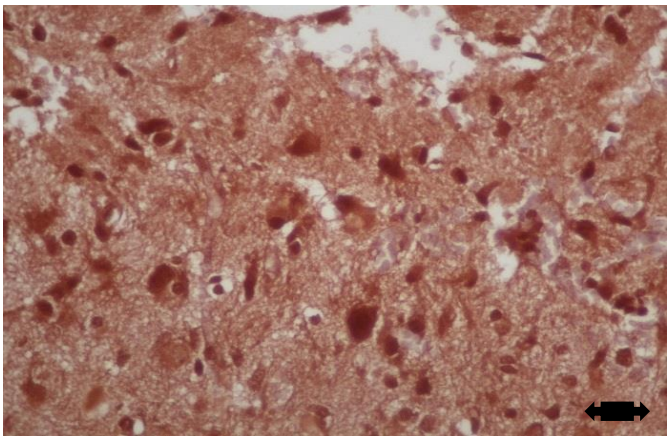
Immunohistochemical analysis in this study demonstrated an increased rate of follistatin immunostaining in low grade gliomas (positive in 66% of specimens tested) than high grade gliomas (positive in 33% of specimens tested). Furthermore, the low grade gliomas exhibited +++ immunostaining in all that were positive whereas in high grade gliomas the intensity of staining in positive specimens was only +. Figure 3.6 (h) is a typical example of +++ immunostaining in a low grade glioma section. There were also differences in the pattern of staining between low and high grade gliomas with the low grade gliomas displaying a predominately interstitial stain with axonal staining. The high grade gliomas displayed a wider variety of staining patterns with both interstitial and cytoplasmic staining. In both grades of glioma nuclear staining was absent. All 6/6 non-cancerous specimens were uniformly negative in nuclear, interstitial and cytoplasmic staining.

3.5 Immunohistochemistry of leptin

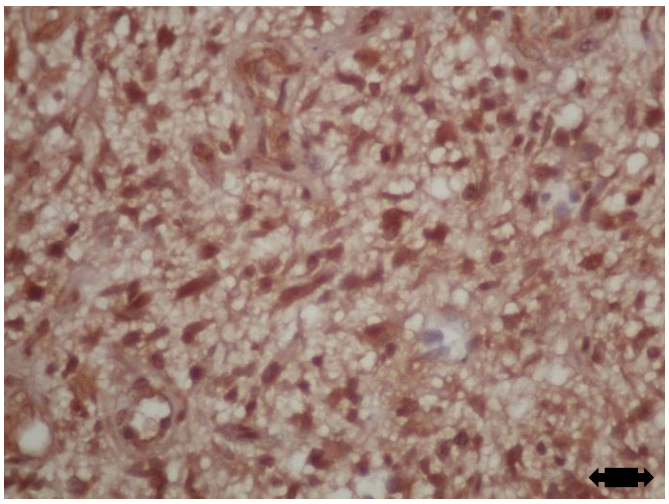
Leptin was found to be neither significantly increased or decreased in the serum of glioma patients. The aim of this study was to determine the likely source of production, of the potential biomarker leptin and to determine if there were any differences in the pattern of expression between primary low and high grade glioma tissue specimens. Immunohistochemical staining of Leptin in 13 high grade glioma, 4 low grade glioma and 4 non-cancerous brain tissue samples was performed according to the protocol described in chapter 2. Testis and ovarian tissue were stained as a negative control. The stained specimens were graded as outlined in chapter 2 according to the intensity of staining in the nucleus, cytoplasm or interstitial space (table 3.12).

3.6 Results of leptin staining

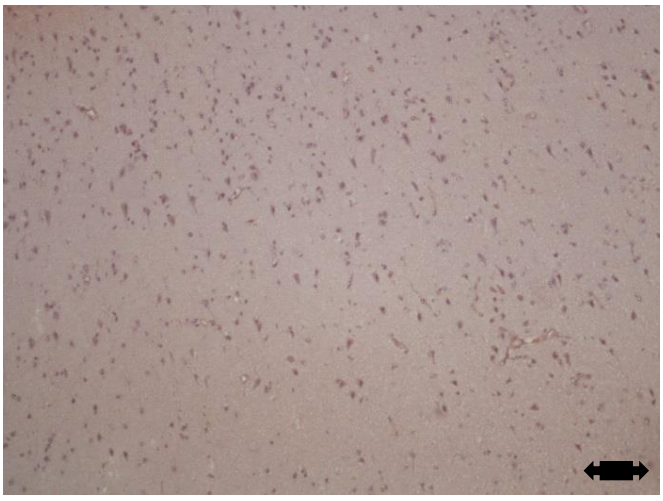
a)



b)



c)



c)

Figure 3.7 **Original photographs showing leptin staining in, a) high grade b) low grade glioma and c) non-cancerous brain sections, mag x40 (a and b) x10 (c) Typical staining of 13 high grade, 4 low grade and 4 non-cancerous sections. Black scale bar represents 20 microns (a and b) and 200 microns (c).**

Table 3.12 The grading of leptin expression in high grade glioma, low grade glioma, non-cancerous brain, testicular and ovarian tissue from immuno-histochemical staining.

BTNW No.	Malignancy Type	Nucleus	Cytoplasm	Interstitial Space
1044	Low Grade	-	+	+
1028	Low Grade	-	+ +	+
934	Low Grade	-	+ +	+ +
1005	Low Grade	+	+ +	+
NB 288/11	High Grade	-	++	+
NB 361/11	High Grade	-	+ + +	+ +
NB 431/11	High Grade	-	+	+
NB 250/11	High Grade	-	+ + +	+ +
NB 302/11	High Grade	-	+	+
NB 293/11	High Grade	-	+ +	+ +
NB 767/11	High Grade	-	+	+
NB 505/11	High Grade	-	+ +	+ +
NB 570/11	High Grade	-	+	+
NB 568/11	High Grade	+	+ + +	+ + +
NB 24/12	High Grade	-	+	+
NB 815/11	High Grade	-	+	+
NB 576/11	High Grade	-	+	+
Testis	Normal Tissue	-	-	+
Ovary	Normal Tissue	-	-	-
Brain 1	Normal Tissue	-	-	+
Brain 2	Normal Tissue	-	-	+
Brain 3	Normal Tissue	-	-	+
Brain 4	Normal Tissue	-	+	+

The results of the study have clearly shown that the expression of leptin was observed in the cytoplasm and in the interstitial spaces of tumour cells of both grades (figures 3.7a and b) with little staining observed in normal brain tissue (figure 3.7c).

High grade glioma specimens displayed predominately, (12/13), cytoplasmic and interstitial staining with no nuclear localisation. In addition, 1/13 specimens displayed weak (+) nuclear localisation. Interstitial staining was graded as (+) for 8/12 of these specimens, (++) for 4 specimens and (+++) for 1 specimen.

Cytoplasmic staining was (+) for 6 specimens, (++) for 4 specimens and (+++) for 3 specimens (table 3.12).

Low grade glioma specimens displayed cytoplasmic and interstitial staining and nuclear localisation of less intensity than the high grade gliomas. There was no nuclear localisation in 3/4 specimens and (+) localisation in 1/4. All of the specimens (4/4) displayed (+) interstitial staining. 3/4 specimens displayed (++) cytoplasmic staining with 1/4 displaying (+) cytoplasmic staining. The main site of leptin production in low grade gliomas was the cytoplasm.

The results also show that non-cancerous brain specimens displayed very little localisation in the nucleus, interstitial space and cytoplasm. All, (4/4), specimens were immuno-negative in the nucleus whilst they were all, (4/4), (+) in the interstitial space. In the cytoplasm 1/4 specimens were (+) and 3/4 were immuno-negative. Non-cancerous ovarian and testicular tissue was used as a negative control in this experiment.

3.7 Immunohistochemistry of Prolactin

The serum levels of prolactin in both low and high grade gliomas were significantly raised in comparison to non-cancerous controls. The aim of this study was to determine if this increase was matched by increased expression in primary tissue. This study analysed 12 high grade, 6 low grade and 6 non-cancerous specimens. The stained specimens were graded as 1, 2 or 3 according to the intensity of staining in the nucleus, cytoplasm or interstices using the scoring system outlined in chapter 2.

3.8 Results of prolactin staining

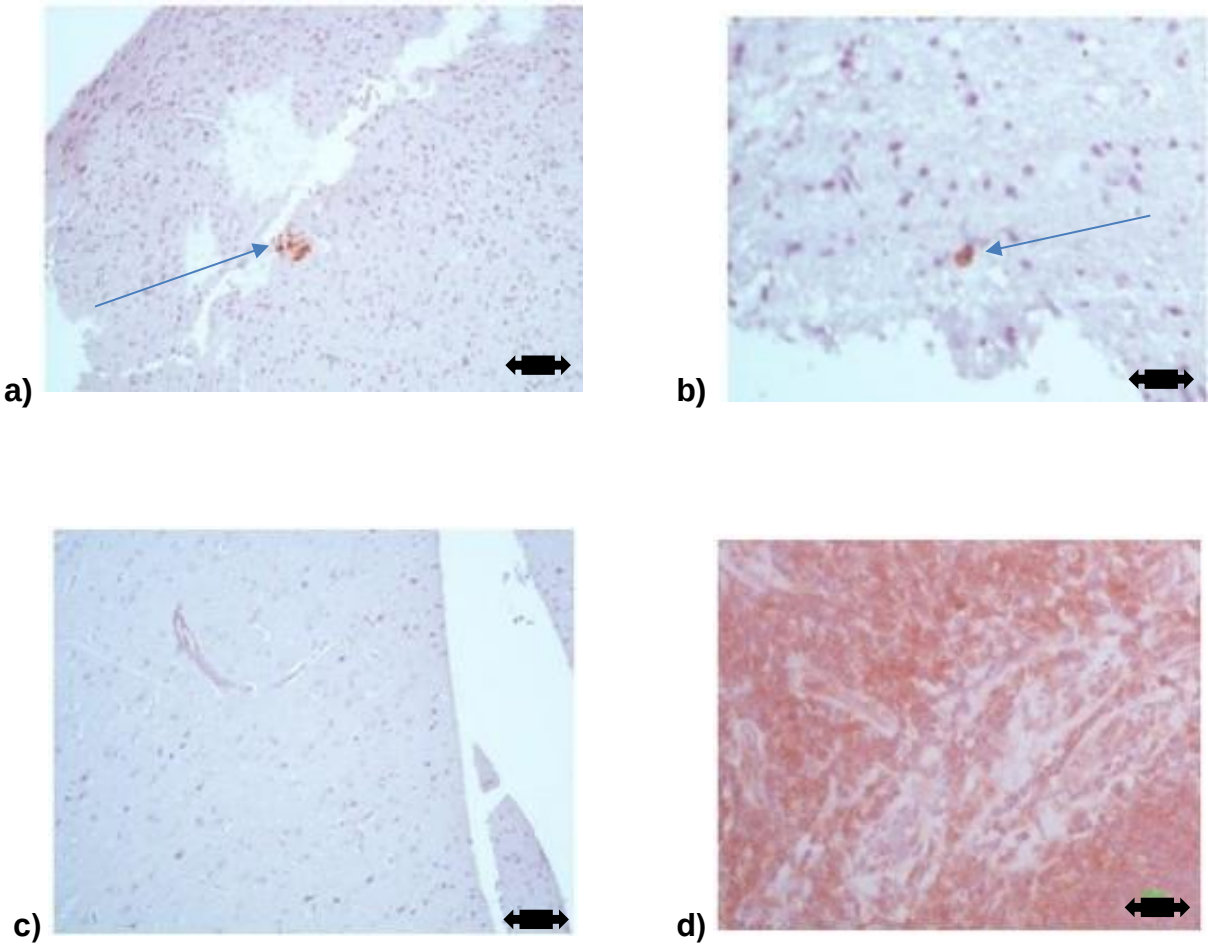


Figure 3.8 Original photographs showing prolactin staining in, a) low grade glioma mag x10, b) high grade glioma mag x40, c) non-cancerous brain mag x10 and d) normal breast tissue mag x40. Typical staining of 12 high grade, 6 low grade and 6 non-cancerous sections. Arrows point to areas of staining. Black scale bar represents 200 microns (a and c) and 20 microns (b and d).

The expression of prolactin was observed in 1/6 (16.7%) low grade glioma samples (Figure 3.8a) and in 3/12 (25%) high grade glioma samples (figure 3.8b). In both grades all staining was weak with a staining intensity score of 1. The staining was cytoplasmic only with no interstitial staining or nuclear localisation. Non-cancerous control specimens (figure 3.8c) were all uniformly negative in nuclear, interstitial and cytoplasmic staining. Normal breast tissue

(figure 3.8d) was stained alongside the tumour and non-cancerous sections and was strongly positive with a staining intensity score of 3 for cytoplasmic and interstitial staining and nuclear localisation.

3.9 Immunohistochemistry of PDGF

The study analysed 18 high grade and 6 non-cancerous specimens. The sections were stained with the following primary antibodies: PDGFRα, PDGF-AA, PDGF-BB and PDGFRβ.

The stained specimens were graded as 1, 2 or 3 according to the intensity of staining in the nucleus, cytoplasm or interstices using the scoring system outlined in chapter 2.

3.10 Results of PDGF staining

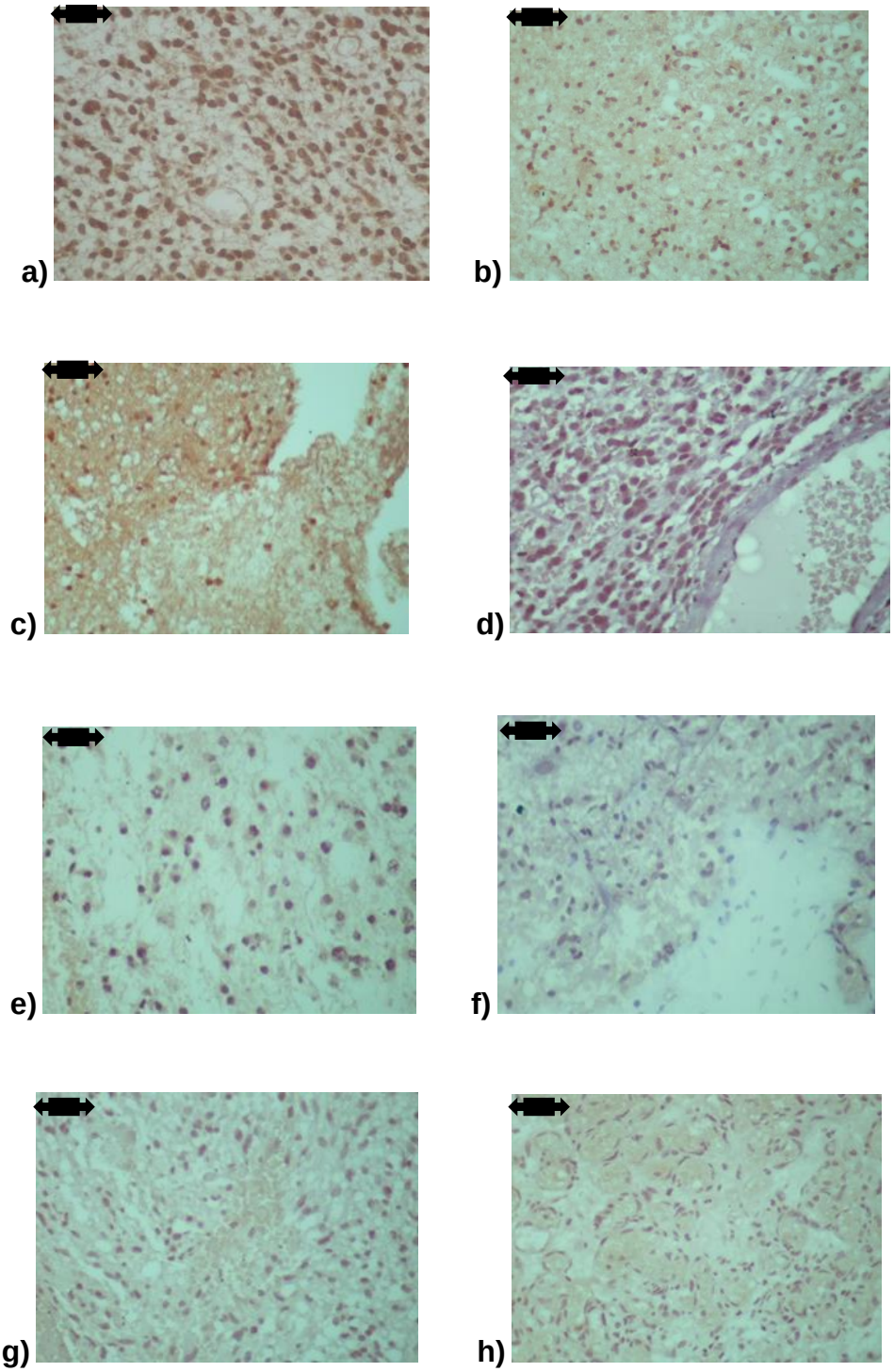


Figure 3.9 **Original photographs showing the expression profile of a single high grade patient for PDGF AA (figure 3.9a), PDGFRα (figure 3.9b), PDGF BB (figure 3.9c) and PDGFRβ (figure 3.9d) and a non-cancerous control for PDGF AA (figure 3.9e), PDGFRα (figure 3.9f), PDGF BB (figure 3.9g) and PDGFRβ (figure 3.9h). Black scale bar represents 20 microns.**

The results show that 93.75% of GBM patients displayed increased PDGFRα expression compared to the non-cancerous controls. In addition, 75% of GBM patients displayed increased PDGF-BB expression and 43.75% of GBM patients displayed increased PDGF-AA expression. However, 56.25% of GBM patients displayed decreased expression of PDGFRβ. The staining was mostly cytoplasmic although 2 patients displayed some interstitial staining (see figure 3.9c). Staining intensity was strong with a score of 3. A total of 6 primary GBM patients underwent both immunohistochemistry ligand staining and serum analysis. Table 3.13 shows the staining score for patients and corresponding serum concentration.

3.11 Correlation between PDGF protein expression and serum composition in primary glioblastoma.

Table 3.13 showing staining score and corresponding serum concentration

BTNW no.	Serum concentration (pg/ml)		Staining score			
	PDGF-AA	PDGF-BB	PDGF-AA	PDGF-BB	PDGFRα PDGFRβ	
1122	28590	342.3	2	2	2	1
511	32180	395.1	2	3	3	3
1154	30440	832.7	3	3	3	2
375	27360	968.2	2	2	3	3
1156	28370	779.9	2	3	2	2
1121	21610	570.1	3	1	3	1

As the results for both serum levels and tissue expression of the PDGF family for 6 patients were obtained it was possible to determine whether there was any significant correlation between the groups. The results show that there was a significant correlation between PDGF-BB ligand expression and PDGF-AA

serum concentration ($r=0.89$, $p<0.05$). There was also some correlation between PDGFRα and PDGF-AA ligand expression ($r=0.5$, $p<0.05$). Please note the discrepancy in serum concentration results with PDGF-BB displayed in figure 3.5. This results from taking measurements using different methodologies, (i.e. Luminex and ELISA). The results are dependent upon the calibration of the instruments and the specificities of the reagents and reference standards used.

3.12 Evaluation of serum and CSF biomarkers for glioma

This study aimed to evaluate the clinical usefulness of an array of potential biomarkers in serum and CSF by comparing the levels of the marker between non-cancerous, high grade glioma and low grade glioma patients.

3.13 Study Protocols

This work comprised of four protocols and they included the following;

1. Comparison of 8 analytes associated with inflammation in 6 patients before and during anaesthetic administration.
2. Comparison of 34 analytes in 44 low grade, 115 high grade and 33 control serum samples. Survival data were obtained for 53 of the high grade patients and a comparison with cytokine levels for each measured significant analyte was made.
3. Comparison of 34 analytes in 8 high grade and 8 control CSF samples
4. Comparison of 34 analytes in 59 high grade pre-debulking serum samples, 50 high grade post debulking samples, 14 control pre-operative samples and 19

control post-operative samples. The control operations were for invasive surgery for non-cancerous conditions using the same anaesthetic protocol.

3.14 Study 1. Anaesthetic effect

Samples were taken both before and during anaesthetic administration. Initially a study was performed to detect whether the anaesthetic drugs had an effect on circulating cytokine levels. This involved taking blood samples from 6 patients before anaesthetic was given as well as during the operation from those same patients whilst under anaesthetic. The 8 analytes chosen for this study reflected the most reactive cytokines traditionally associated with the inflammatory response. These were IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF-γ and TNF-α. None of the 6 paired human serum cytokine concentrations demonstrated a significant difference ($p>0.05$) between pre-anaesthetic levels and post-anaesthetic levels as shown in Table 3.14.

Table 3.14. The mean cytokine concentrations in pg/ml between paired samples taken before and after general anaesthetic.

Cytokine		Mean pg/ml	N	Paired differences pg/ml	Significance (p<0.05)
Pair 1	IL-2 Preanaesthetic	2307.91	6		
	IL-2 Postanaesthetic	2301.41	6	6.5	No
Pair 2	IL-4 Preanaesthetic	3562.32	6		
	IL-4 Postanaesthetic	3559.21	6	3.11	No
Pair 3	IL-6 Preanaesthetic	3186.57	6		
	IL-6 Postanaesthetic	3183.67	6	2.9	No
Pair 4	IL-8 Preanaesthetic	1273.02	6		
	IL-8 Postanaesthetic	1271.81	6	1.21	No
Pair 5	IL-10 Preanaesthetic	4838.82	6		
	IL-10 Postanaesthetic	4836.32	6	2.5	No
Pair 6	GM-CSF Preanaesthetic	2523.87	6		
	GM-CSF Postanaesthetic	2520.41	6	3.46	No
Pair 7	IFN-γ Preanaesthetic	1801.05	6		
	IFN-γ Postanaesthetic	1800.99	6	0.06	No
Pair 8	TNF-α Preanaesthetic	3156.07	6		
	TNF-α Postanaesthetic	3155.83	6	0.24	No

3.15 Study 2. Comparison of 44 low grade, 115 high grade and 33 control serum samples.

Study 1 established that anaesthetic given to glioma patients did not affect the production of the most reactive cytokines. This enabled the collection of pre and post-operative samples without the need to account for any anaesthetic effect. Therefore the aim of study 2 was to compare blood serum levels of 34 analytes that have been implicated in other cancers and in angiogenesis to determine if any were suitable as a potential biomarker to aid diagnosis of high and low grade glioma. Tables 3.15 and 3.16 show the concentrations of the

different biomarkers in the analysis. The results clearly show no significant difference between any of the three groups (low grade, high grade and non-cancerous). Figure 3.10 shows that analytes had some significant difference between the three groups.

Table 3.15. Mean values for non-cancerous (n=33) and low grade (n=44) glioma sera samples with no significant difference. These results were generated with those in figure 3.10 and should be read in conjunction with those.

Cytokine		Median pg/ml	Mean -/+ sd pg/m	N	Paired differences of means pg/ml	Significance (p<0.05)
sEGFR	Non-cancerous	17351	17582 -/+ 906	33		
	Low grade glioma	18977	18841 -/+ 904	44	1259	No
HGF	Non-cancerous	3157	3175 -/+ 106	33		
	Low grade glioma	2540	2622 -/+ 94	44	553	No
Osteopontin	Non-cancerous	30345	34092 -/+ 2932	33		
	Low grade glioma	30541	34582 -/+ 2849	44	490	No
SCF	Non-cancerous	452	458 -/+ 12	33		
	Low grade glioma	443	471 -/+ 18	44	13	No
TIE-2	Non-cancerous	12092	12457 -/+ 494	33		
	Low grade glioma	12269	12673 -/+ 467	44	216	No
sVEGFR-2	Non-cancerous	4962	5107 -/+ 268	33		
	Low grade glioma	5309	5574 -/+ 248	44	467	No
sCD40L	Non-cancerous	1157	1193 -/+ 63	33		
	Low grade glioma	1146	1225 -/+ 60	44	32	No
Endoglin	Non-cancerous	1130	1267 -/+ 76	33		
	Low grade glioma	1131	1231 -/+ 72	44	36	No
sFASL	Non-cancerous	298	299 -/+ 9	33		
	Low grade glioma	309	318 -/+ 7	44	19	No
HB-EGF	Non-cancerous	56	58 -/+ 2	33		
	Low grade glioma	61	62 -/+ 2	44	4	No
IGFBP-1	Non-cancerous	7174	14104 -/+ 2535	33		
	Low grade glioma	9241	14127 -/+ 1885	44	23	No
IL-6	Non-cancerous	50	65 -/+ 10	33		
	Low grade glioma	45	54 -/+ 9	44	11	No
IL-8	Non-cancerous	18	20 -/+ 2	33		
	Low grade glioma	16	16 -/+ 1	44	4	No
IL-18	Non-cancerous	214	235 -/+ 16	33		
	Low grade glioma	205	251 -/+ 30	44	16	No
PAI-1	Non-cancerous	189738	228010 -/+ 22587	33		
	Low grade glioma	199743	262072 -/+ 28253	44	34062	No
PLGF	Non-cancerous	83	85 -/+ 3	33		
	Low grade glioma	86	85 -/+ 3	44	0	No
TGF-α	Non-cancerous	92	100 -/+ 6	33		
	Low grade glioma	98	99 -/+ 4	44	1	No
TNF-α	Non-cancerous	32	31 -/+ 1	33		
	Low grade glioma	32	32 -/+ 1	44	1	No
VEGF-A	Non-cancerous	709	740 -/+ 61	33		
	Low grade glioma	719	722 -/+ 42	44	18	No
VEGF-C	Non-cancerous	1926	1914 -/+ 98	33		
	Low grade glioma	2032	2136 -/+ 114	44	222	No
VEGF-D	Non-cancerous	887	887 -/+ 15	33		
	Low grade glioma	903	881 -/+ 17	44	6	No

Table 3.16. Mean values for non-cancerous (n=33) and high grade (n=115) glioma sera samples with no significant difference. These results were generated with those in figures 3.10 and should be read in conjunction with those.

Cytokine		Median pg/ml	Mean -/+ sd pg/ml	N	Paired differences pg/ml	Significance (p<0.05)
sEGFR	Non-cancerous	17351	17582 -/+ 906	33		
	High grade glioma	18145	18047 -/+ 453	115	465	No
HGF	Non-cancerous	3157	3175 -/+ 106	33		
	High grade glioma	2708	2712 -/+ 65	115	463	No
Osteopontin	Non-cancerous	30345	34092 -/+ 2932	33		
	High grade glioma	31343	35907 -/+ 1948	115	1815	No
SCF	Non-cancerous	452	458 -/+ 12	33		
	High grade glioma	454	435 -/+ 10	115	23	No
TIE-2	Non-cancerous	12092	12457 -/+ 494	33		
	High grade glioma	11550	11367 -/+ 273	115	1090	No
sVEGFR-2	Non-cancerous	4962	5107 -/+ 268	33		
	High grade glioma	5234	5255 -/+ 139	115	148	No
sCD40L	Non-cancerous	1157	1193 -/+ 63	33		
	High grade glioma	1108	1178 -/+ 40	115	15	No
Endoglin	Non-cancerous	1130	1267 -/+ 76	33		
	High grade glioma	1071	1154 -/+ 41	115	113	No
sFASL	Non-cancerous	298	299 -/+ 9	33		
	High grade glioma	308	308 -/+ 3	115	9	No
HB-EGF	Non-cancerous	56	58 -/+ 2	33		
	High grade glioma	62	68 -/+ 2	115	10	No
IGFBP-1	Non-cancerous	7174	14104 -/+ 2535	33		
	High grade glioma	10180	17300 -/+ 1521	115	3196	No
IL-6	Non-cancerous	50	65 -/+ 10	33		
	High grade glioma	43	46 -/+2	115	19	No
IL-8	Non-cancerous	18	20 -/+ 2	33		
	High grade glioma	15	19 -/+ 3	115	1	No
IL-18	Non-cancerous	214	235 -/+ 16	33		
	High grade glioma	175	207 -/+ 12	115	28	No
PAI-1	Non-cancerous	189738	228010 -/+ 22587	33		
	High grade glioma	212391	236085 -/+ 10559	115	8075	No
PLGF	Non-cancerous	83	85 -/+ 3	33		
	High grade glioma	80	79 -/+ 1	115	6	No
TGF-α	Non-cancerous	92	100 -/+ 6	33		
	High grade glioma	98	99 -/+ 2	115	1	No
TNF-α	Non-cancerous	32	31 -/+ 1	33		
	High grade glioma	30	30 -/+ 1	115	1	No
VEGF-A	Non-cancerous	709	740 -/+ 61	33		
	High grade glioma	737	769 -/+ 26	115	29	No
VEGF-C	Non-cancerous	1926	1914 -/+ 98	33		
	High grade glioma	2022	2093 -/+ 57	115	179	No
VEGF-D	Non-cancerous	887	887 -/+ 15	33		
	High grade glioma	856	854 -/+ 7	115	33	No

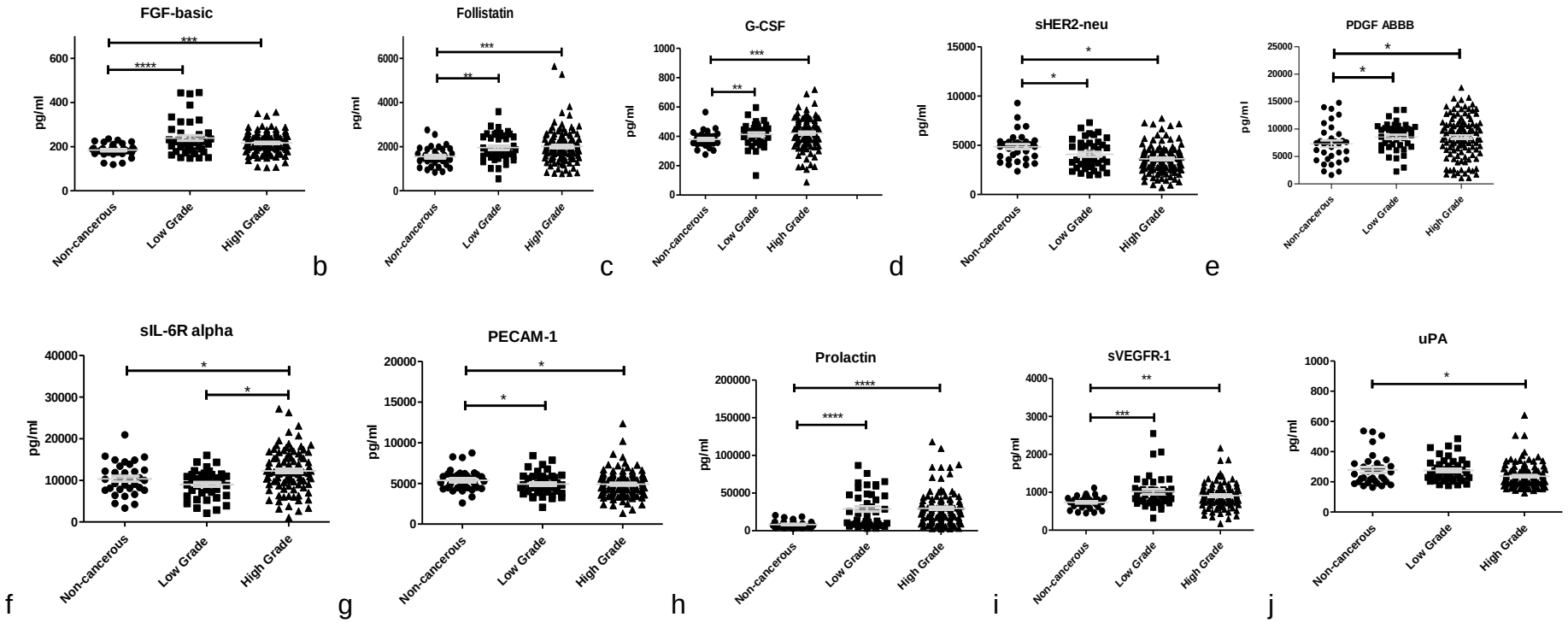


Figure 3.10 Scatterplots and table displaying the concentrations of 10 analytes including; a) FGF-basic, b) Follistatin, c) G-CSF, d) sHER2-neu, e) PDGF ABBB, f) sIL-6Ralpha, g) PECAM-1, h) Prolactin, i) sVEGFR-1, j) uPA in sera taken from patients with low and high grades of glioma compared to a non-cancerous group. The significant differences * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 between non-cancerous, low grade and high grade glioma sera are shown above the horizontal bars. The means and standard deviations are shown in grey for comparison. n= 33 for non-cancerous, 44 for low grade and 115 for high grade samples. These results are also shown in table 3.17.

Table 3.17 displaying the results from figure 3.10 in numerical form.

Cytokine	Non-cancerous			Low grade			High grade		
	Mean pg/ml	sd	n	Mean pg/ml	sd	n	Mean pg/ml	sd	n
FGF-basic	184.6	4.9	33	239	12.5	44	216.6	4.5	115
Follistatin	1548.4	78.4	33	1954	93.7	44	2009.7	71.3	115
G-CSF	379.4	9.4	33	413.1	13.2	44	435.4	16.2	115
sHer2-neu	4845.3	252	33	4079.5	227.2	44	3615.8	13.4	115
PDGF-ABBB	7315.1	603.9	33	8572.4	391.9	44	8536.8	355.5	115
sIL-6R alpha	10352.8	669.4	33	9027.9	507.8	44	12242.3	455	115
PECAM-1	5382.4	236.7	33	4918	215	44	4907.3	147.4	115
Prolactin	8101.9	802.9	33	28875.1	3667.8	44	32603.8	3541.9	115
sVEGFR-1	736.6	27.2	33	1017.6	65.5	44	911.2	28.7	115
uPA	278.4	18.1	33	275.8	11.5	44	236.9	7.2	115

The data presented in figure 3.10 and table 3.17 show a variety of differences in serum levels of the potential biomarkers. 7/10 of these biomarkers including FGF-basic, Follistatin, G-CSF, PDGF ABBB, sIL-6Rα, Prolactin and sVEGFR-1, showed increasing levels of the analyte in low and high grade glioma sera compared with the non-cancerous controls. In contrast 3/10, which included sHER2-neu, PECAM-1 and uPA showed decreasing levels in comparison. Several of the measured analytes including Prolactin, sVEGFR-1, FGF-basic, Follistatin, G-CSF, SHER2-neu, sIL-6Rα and uPA show potentials as diagnostic biomarkers. As such they seem to be promising candidates for diagnostic tools in the detection of glioma. Interestingly sIL-6Rα and uPA were able to distinguish between low and high grade gliomas, however, all of the potential biomarkers must go through the confirmation, assay development and validation stages of biomarker approval. Cancer Research UK have produced an indicative roadmap for a new biomarker validation that incorporates the above stages (Cancer Research UK, 2017).

Statistical power analysis is a measure of the probability of rejecting a null hypothesis when the alternative hypothesis is true. In terms of this study it is a measure of the ability of each biomarker to predict the presence of a brain tumour. The mean and standard deviation (SD) of the non-cancerous, high-grade and low-grade cohorts was calculated for each biomarker. An individual patient was considered positive if their result was above or below the mean of the non-cancerous cohort plus or minus 2SD depending whether the biomarker was raised or lowered in the glioma cohort compared to the non-cancerous control. Power analysis of the data in this study indicates that prolactin (100%), sVEGFr-1 (99.99%), FGF (99.8%), follistatin (99.6%), G-CSF (99.6%) and sHer2-neu (99%) are the strongest performing potential biomarkers. The specificity and sensitivity levels are summarised in Table 3.18. This study had sufficient sample sizes to demonstrate 80% power in 5 potential biomarkers with sHER2-neu being just outside of this sample size range. Moreover, sHER2-neu levels showed a decreasing gradation of levels between non-cancerous, low grade and high grade sera. The specificity for distinguishing between low and high grade glioma was 51% for this analyte.

Table 3.18 The sensitivity and specificity of potential biomarkers in measuring serum levels of several analytes to distinguish sera from low grade and high grade glioma patients from sera from non-cancerous patients. All data are expressed as a percentage

Analytes	LGG		HGG		Power	Sample size for 80% power
	Sen.	Spec.	Sen.	Spec.		
Prolactin	70%	100%	83%	100%	100%	2
sVEGFr-1	59%	67%	69%	67%	99.99%	28
FGF	73%	82%	72%	82%	99.80%	28
Follistatin	64%	70%	70%	70%	99.60%	34
G-CSF	77%	67%	65%	67%	99.60%	32
sHER2-Neu	59%	64%	76%	64%	99.00%	49

These data presented in table 3.18 were obtained by producing a reference range from the non-cancerous control data using a 95% confidence interval calculated by using the mean plus and minus 2 standard deviations. Individuals with levels outside of these ranges were considered abnormal and were deemed positive for the disease diagnosis depending whether the analyte is raised or lowered in the diseased cohort. The sensitivity of the biomarker is a measure of the ability of the biomarker to correctly measure the proportion of positives identified as such. This is also known as the true positive rate. The specificity of the biomarker measures the proportion of negatives identified as such also known as the true negative rate.

Figure 3.11 shows the survival of high grade glioma patients from first diagnosis. The data showed that almost 80% of patients with high grade glioma

died within 20 months of first diagnosis. By 5 years after diagnosis all patients had died.

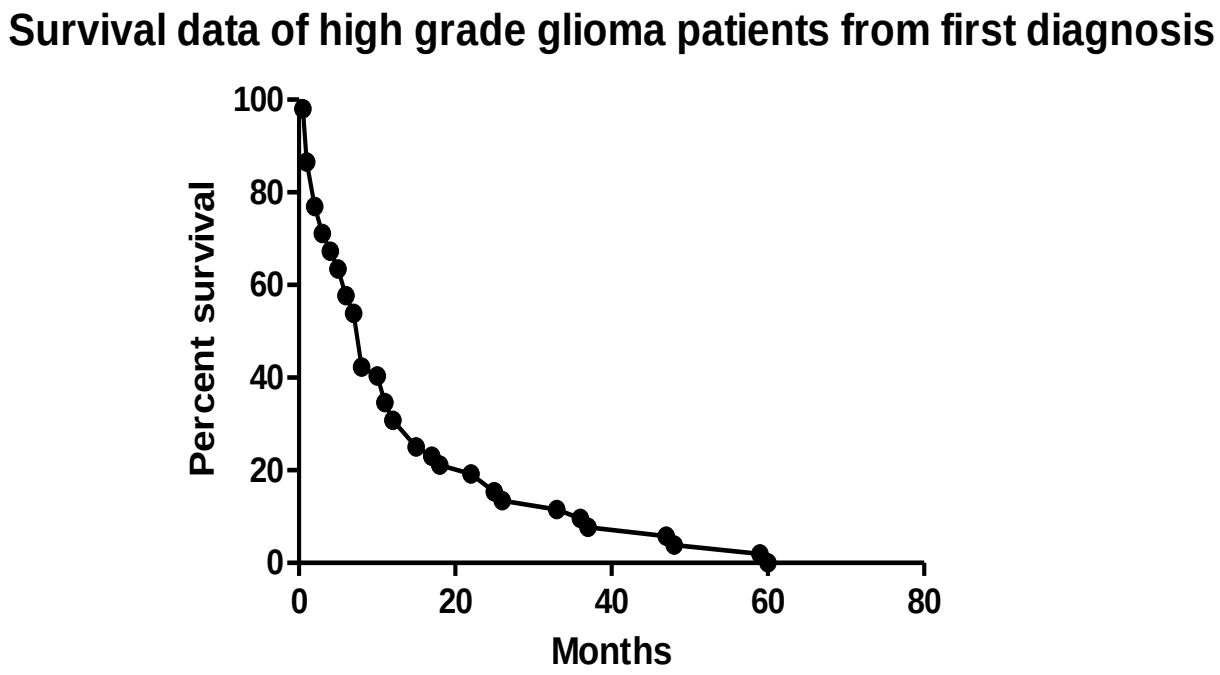


Figure 3.11. Kaplan-Meier plot of survival for 53 high grade glioma samples

Table 3.19 shows that there was no correlation with the levels of any analyte with either survival or gender.

Table 3.19 The correlation between the level of the potential biomarker (pg/ml) and survival in months (r) and the probability (p) of a correlation in gender (male or female). Correlation coefficient r > +/- 0.5 and p <0.05 to be considered correlated. See Appendix A for data set.

Analytes	Correlation with survival	Survival r value	Correlation with gender (male or female)	Gender p value
Prolactin	None	0.279	None	0.08
sVEGFr-1	None	-0.031	None	0.23
FGF - basic	None	0.035	None	0.36
Follistatin	None	0.025	None	0.39
G-CSF	None	-0.048	None	0.64
sHER2-Neu	None	0.083	None	0.24
sIL-6R alpha	None	-0.036	None	0.33
PECAM-1	None	0.005	None	0.61
uPA	None	-0.127	None	0.05
PDGF-ABBB	None	0.191	None	0.41

3.16 Study 3. Measurements of potential biomarkers in 8 high grade and 8 control CSF samples

The aim of the third part of the study was to determine whether Cerebro-Spinal Fluid (CSF) could also be used either in place of or alongside blood serum as a source of potential diagnostic biomarkers for high grade glioma. A further aim was to determine if the same analytes that were significantly different in blood serum were also significantly different in CSF. The data presented in figure 3.12 and table 3.20 showed that there was some correlation between those biomarkers identified as being significantly, ($p<0.05$) dysregulated in sera and those biomarkers identified as significantly different in CSF. In addition, other significantly, ($p<0.05$) dysregulated biomarkers were detected in the CSF that were not identified as dysregulated in the sera. These were sEGRF and SCF. (figure 3.12).

Table 3.20 Mean values for non-cancerous (n=8) and high grade (n=8) glioma CSF samples with no significant difference. These results were generated with those in figures 3.12 and should be read in conjunction with those.

Cytokine		Mean (pg/ml)	standard deviation	n	paired differences	significance p<0.05
FGF-basic	non-cancerous	121.6	52	8	63.1	
	high grade	184.7	27.9	8		no
sHer2- neu	non-cancerous	507.1	203.3	8	281.3	
	high grade	788.4	176.6	8		no
HGF	non-cancerous	1313.7	511	8	271.9	
	high grade	1041.8	308.3	8		no
Leptin	non-cancerous	555.6	308.3	8	44.7	
	high grade	510.9	359.1	8		no
Prolactin	non-cancerous	1906.3	723.6	8	-794.1	
	high grade	2700.4	246.5	8		no
PDGF- ABBB	non-cancerous	170.3	86.7	8	-113	
	high grade	283.3	69.8	8		no
Osteopontin	non-cancerous	44125.3	23420.5	8	30569.9	
	high grade	13555.4	21761.2	8		no
TIE-2	non-cancerous	3708.4	2035	8	-2784.1	
	high grade	6492.5	1665	8		no
sVEGFR-2	non-cancerous	2504	1515.7	8	-1493.6	
	high grade	3997.6	548.6	8		no
sCD40L	non-cancerous	387.4	117.3	8	161.1	
	high grade	226.3	79.1	8		no
Endoglin	non-cancerous	488.2	127	8	51.9	
	high grade	436.3	217.3	8		no
sFASL	non-cancerous	257.6	176.3	8	95.8	
	high grade	161.8	70.2	8		no
HB-EGF	non-cancerous	36.3	7.6	8	5.1	
	high grade	31.2	5.1	8		no
IGFBP-1	non-cancerous	9090.1	5829.5	8	4799.5	
	high grade	4290.6	1748.7	8		no
IL-6	non-cancerous	351.7	320.4	8	308.2	
	high grade	43.5	36.7	8		no
IL-8	non-cancerous	1757.6	2386.7	8	1483.6	
	high grade	274	472.1	8		no
IL-18	non-cancerous	52.4	18.9	8	-16.4	
	high grade	68.8	46.6	8		no
PAI-1	non-cancerous	24225	16028.5	8	-9218	
	high grade	33443	11596.1	8		no
PLGF	non-cancerous	268.4	53.2	8	17.7	
	high grade	250.7	92.2	8		no
TGF-α	non-cancerous	74.1	18.1	8	19.9	
	high grade	54.2	10.8	8		no
TNF-α	non-cancerous	26	6.4	8	9.5	
	high grade	16.5	3.6	8		no
uPA	non-cancerous	504.4	111.1	8	95.6	no
	high grade	408.8	129.7	8		no
VEGF-A	non-cancerous	747.3	431.8	8	437.2	
	high grade	310.1	98.4	8		no
VEGF-C	non-cancerous	987	236.6	8	299	
	high grade	688	130.6	8		no
VEGF-D	non-cancerous	640.7	109.9	8	121.9	
	high grade	518.8	72.1	8		no

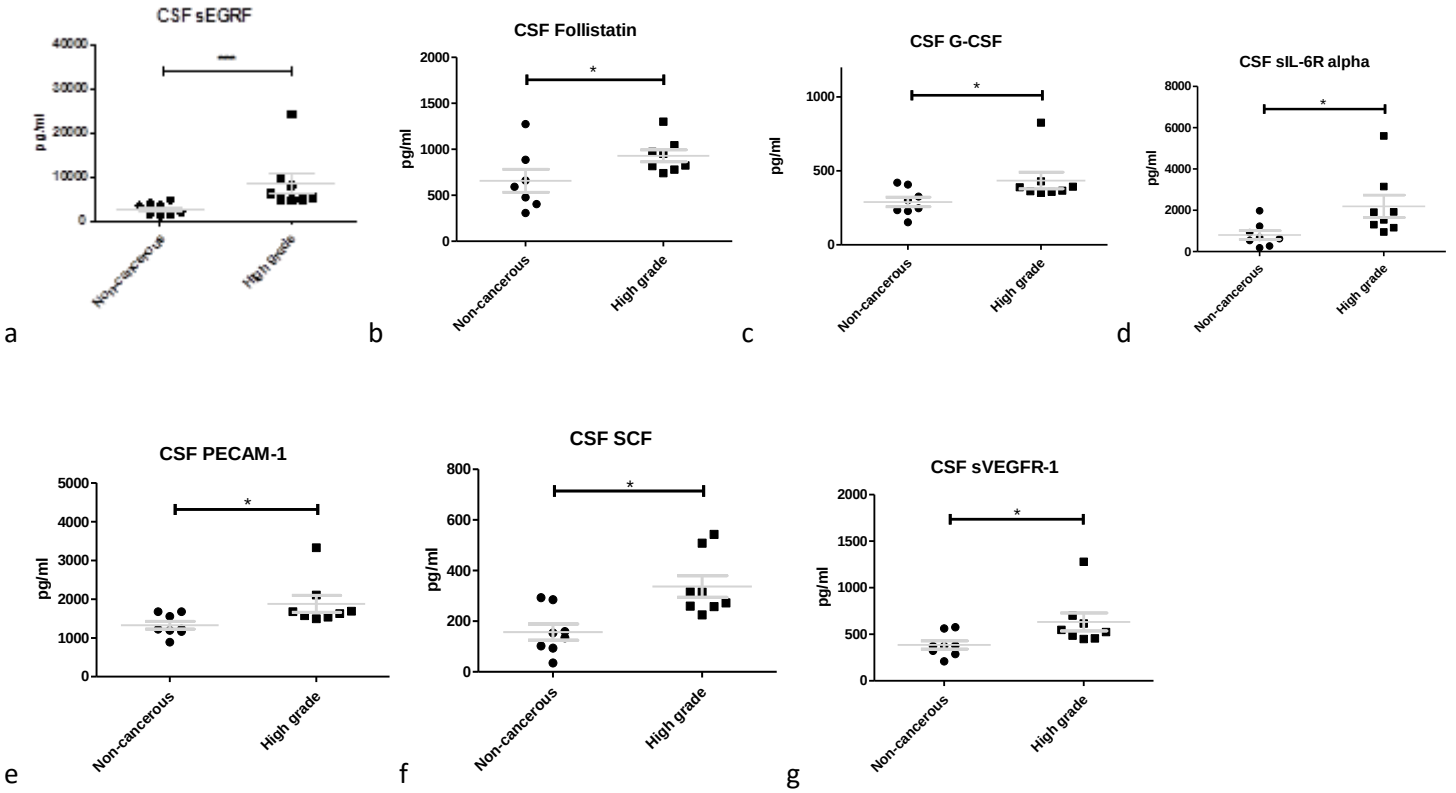


Figure 3.12 Scatterplots displaying the concentrations of 8 analytes namely; a) sEGRF, b) Follistatin, c) G-CSF, d) sIL-6Ralpha, e) PECAM-1, f) SCF, g) sVEGFR-1 in CSF in non-cancerous control and high grade glioma samples. The significant differences * = p<0.05 and *** = p<0.001, between non-cancerous, and high grade glioma CSF samples are shown above the horizontal bars. The means and standard deviations are shown in grey for comparison. n= 8 for non-cancerous and high grade samples. These results are also shown in table 3.21.

Table 3.21 displaying the results from figure 3.12 in numerical form.

cytokine	Non-cancerous			High grade		
	Mean pg/ml	sd	n	Mean pg/ml	sd	n
sEGFR	2740.6	500.7	8	8500.5	2325.9	8
Follistatin	657.5	124.8	8	928.6	64.8	8
G-CSF	289.8	32.7	8	434.7	56.7	8
sIL-6R alpha	802.7	205.2	8	2191.6	543.7	8
PECAM-1	1326	99.6	8	1878.7	217.4	8
SCF	157	32	8	337.1	42.6	8
sVEGFR-1	385	44.8	8	632.2	97.2	8

3.17 Study 4. Measurements of biomarkers in 59 high grade pre-debulking serum samples, 50 high grade post debulking samples, 14 control pre-op samples and 19 control post op samples for comparison.

So far, studies 1-3 in this investigation have shown no significant change in inflammatory cytokines during the administration of general anaesthetic before surgery. However, the data have established significant differences in several analytes between non-cancerous control patients, low grade glioma and high grade glioma patients in serum and CSF. There was also some correlation in sera and CSF of a number of potential biomarkers that were dysregulated in high grade glioma, suggesting that the production of these potential biomarkers were from the same source.

Study 4 aimed to test whether there was a significant change in the levels of these analytes following de-bulking of tumour surgery which would allow for the monitoring of surgical treatment through measuring analyte levels post operatively, as well as providing evidence for the source of production of the circulating biomarker. Of the analytes investigated, Follistatin, G-CSF and

Prolactin showed a significant ($p<0.05$) reduction in detectable levels in the sera of glioma patients after surgery compared to the control. In contrast no significant difference was detected in the sera of non-cancerous patients post-operatively compared to pre-operatively (figure 3.13).

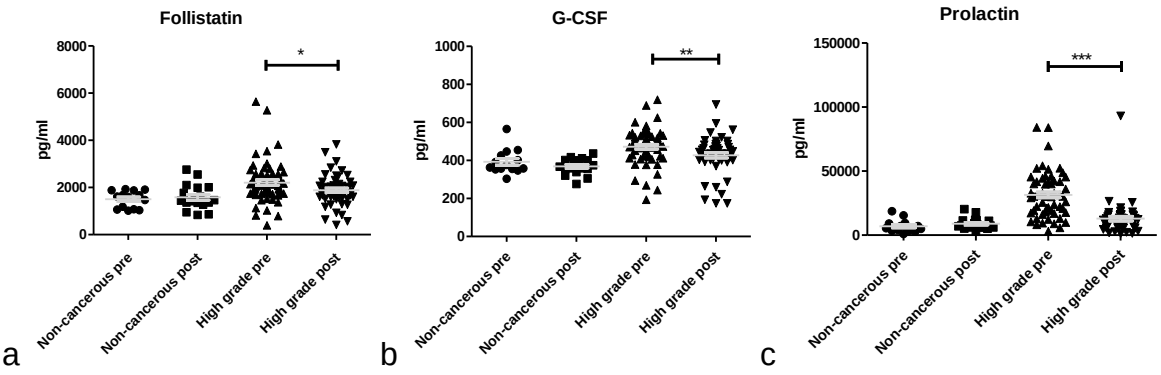


Figure 3.13 Scatterplots displaying the concentrations of 3 analytes namely; a) Follistatin, b) G-CSF and c) Prolactin. The significant differences * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$, between non-cancerous, and high grade glioma serum samples are shown above the horizontal bars. The means and standard deviations are shown in grey for comparison. Each solid, triangle and square represents one sample and n= 59 high grade pre-debulking serum samples, 50 high grade post debulking samples, 14 control pre-operative samples and 19 control post-operative samples. The results are also shown in table 3.22.

Table 3.22 displaying the results from figure 3.13 in numerical form.

cytokine	Non-cancerous				High-grade			
	Pre-op		Post-op		Pre-op		Post-op	
	Mean pg/ml	sd	Mean pg/ml	sd	Mean pg/ml	sd	Mean pg/ml	sd
Follistatin	1501.2	95.1	1583.2	118.4	2204.4	115.7	1881.5	94.4
G-CSF	392.9	17.3	417.3	49.2	497	27.5	427	14.2
Prolactin	6966.4	1312.3	8938.7	991.2	36956.4	5970.9	12903	1843.4

In all 3 analytes, a high pre-operative level in the glioma patient samples reduced to levels that are almost comparative to non-cancerous sera in the post-operative sample. The non-cancerous sera did not show any significant difference between pre and post-operative levels.

Chapter 4

General discussion

4.1 Introduction

The aims of this thesis were twofold. The first was to investigate the role of lymphocyte subsets in the generation of an immunosuppressive microenvironment in glioma patients in order to identify a possible therapeutic target. After an investigation into the optimal method of sample collection without effect on cytokine secretion, it was decided that fresh specimens should be collected where possible. Regulatory T cells were then enumerated (figure 3.3) in patients with both malignant and non-malignant tumours with no difference between the groups being found. The second aim was to characterise the specific cytokine levels measureable in both serum and cerebro-spinal fluid (CSF) in both malignant and non-malignant glioma patients and identifying the source of the specific secretions. These experiments revealed a consistent difference between the groups in the levels of specific analytes in both serum and CSF. The levels were shown to respond to tumour removal and so the utility of measuring these secretions was revealed. Immunohistochemical analysis of tumour sections from these patients showed an increased production of the analytes within the tumour suggesting that the site of production is the tumour itself and not cells in the periphery. These analytes had a potential to act as specific biomarkers for these disorders and a panel of biomarkers was developed and tested for both low and high grade patient serum analysis with the ability to distinguish between low grade, high grade and non-cancerous sera. These panels gave sensitivities and specificities of up to 75% and 76%, respectively although these figures are related to the methodology employed in this analysis and may be different if another methodology were used.

The general discussion will now concentrate on a number of results including the lymphocyte viability study, the presence of Tregs in glioma, immunohistochemistry for follistatin, leptin, and PDGF, comparisons of potential biomarkers in serum and CSF and subsequently a new proposed diagnostic testing strategy.

4.2 Lymphocyte viability study

In this part of the study involving the effect of cryopreservation on lymphocyte viability it was shown that viability was significantly lower in the samples which had been cryopreserved than those which had been stored at 4°C, (table 3.3). These results were contradicted compared to some other reports (Buhl, et al. 2012) but supported by others who showed a loss of T-cell function after long-term cryopreservation (Owen, et al. 2007).

Another interesting result of this study is the lack of negative correlation of viability with length of storage of the sample, (table 3.1). There was no decrease in viability despite the oldest sample being tested 13 days after collection. Interestingly however, this sample contained the highest concentration of TNF-α, suggesting higher levels of apoptosis, which again was not consistent with the high percentage viability.

The media added to samples can significantly alter cell viability. The addition of human AB serum demonstrates a reduced viability when compared with other sera. These researchers also found that the temperature of the washing media can influence the cell viability suggesting that the washing media should be warmed before use (Disis, et al. 2006).

TNF-α was chosen as a product of apoptosis and the increased secretion of which was potentially indicative of this process occurring (Sabri, et al. 2003). The patient with the highest concentration of TNF-α of 84 pg/ml, (table 3.5) was still within normal ranges of 75 pg/ml ± 15 (Damas, et al. 1989). The remaining samples had consistent levels of TNF-α of around 25 pg/ml within the normal range. This observation suggests that for these patients there was no increase in TNF-α in peripheral blood.

4.3 Presence of Tregs in high and low grade gliomas

The buffy coat samples used in this study were stored at -80°C without the use of cryopreservatives such as dimethylsulphoxide (DMSO). These were used as a concentrated source of white blood cells to be used in the analysis. This may have resulted in the excess cellular debris that was evident on the scatterplots from the flow cytometer, (figure 3.3). Gating was performed on the forward scatter-side scatter plot in the region that contained lymphocytes according to the manufacturer's recommendations. Unstained cells were analysed and a degree of autofluorescence was detected which was reduced upon altering the gains and compensation values of the laser in the flow cytometer. There was also significant interference from other cell types and haemoglobin contamination making separation of the cell pellet difficult.

In this current work, the mean percentage of CD4+CD25+FoxP3+ Tregs was determined in both glioblastoma and meningioma peripheral patient buffy coat samples. The results showed that the percentage of Tregs in GBM patients was slightly lower (1.72 ± 0.233%) when compared to meningioma patients (2.35 ± 0.85%), (table 3.9). There was no significant difference between the two groups with p=0.418. Fecci, et al. (2006) reported a diminished fraction of

CD4+CD25+FoxP3+ Tregs in patients with malignant glioma but they further suggested that they also formed an increased percentage of the CD4 compartment which was sufficient to cause the decreased T-cell function found in these patients. The lower proportion of Tregs in both malignant and non-malignant tumours suggests that any role these cells may play is not connected to the degree of malignancy, angiogenesis and proliferation of these tumours.

The present data show clearly a significant difference in the viability of cells from the fresh and frozen samples with significantly decreased lymphocyte viability observed after cryopreservation. The findings suggest that this method of cryopreservation may not be suitable for further experimental models, and that freshly isolated lymphocyte samples only should be used to detect secreted cytokines and apoptotic proteins. Further research into the role of TNF-α as an apoptotic factor in lymphocytes would be beneficial (Bao and Cao 2014).

The Treg enumeration experiment involved the transport of buffy coat samples from the BTNW tissue bank. Mallone, et al. (2011) indicated that transportation conditions can affect the quality and characteristics. Other researchers have suggested that specific alterations in the CD4+/CD8+ ratio and the secretion of PBMC cytokines are altered depending on the storage and transportations conditions whilst NK cell and B cell subsets retain their phenotype (Posevitz-Fejfar, et al. 2014). There was little or no data in the literature concerning the use of buffy coat samples but the decision was taken to use them as a large number of samples could be transported in one trip in a polystyrene transport box with frozen ice packs to maintain a temperature of at least -20°C.

The samples collected showed a disparity in the age ranges for the two groups. The glioblastoma patients' age ranges were from 58-85 years whilst the meningioma age ranges were from 24-71 years. Previous research studies have suggested that the Treg compartment increases with age (Garg, et al. 2014) and so future studies should be age matched to improve the accuracy of the comparison.

The method of thawing of the frozen buffy coat samples was investigated with two methods tested for the effect on cell viability, slow (at 4°C) and fast (at 37°C), (tables 3.6 and 3.7). Cells that had been thawed slowly had a higher average viability (70.01%) than those that had been thawed quickly (55.64%). This was the reason for thawing at 4°C. However, the results show a wide variability of viabilities in the fast thawing method (8.8%-95%). This may be due to the prior treatment of the buffy coat samples at the Royal Preston hospital before they were collected.

The cytokine analysis was performed on frozen serum samples received from the BTNW tissue bank stored at -80°C. These samples were transported to the research laboratories in polystyrene boxes with frozen ice packs inserted. They were transferred directly to the -80°C freezer upon arrival at UCLan, Preston, UK. Thawing took place at room temperature and once the samples had been aliquoted they were immediately snap frozen in liquid nitrogen.

Patients affected by malignant gliomas in this study had, in most cases, higher pre-operative concentrations of analyte than the non-cancerous controls. The exceptions to this were IL-8 and Angiopoietin-2, (figure 3.5), in which the control group had a significantly higher concentration than the glioma group. Gliomas

produce angiogenesis factors in response to hypoxia while promoting neo-angiogenesis and growth (Yano, et al. 2006). The patients in this study were taking dexamethasone (16 mg/day), which has been shown to downregulate the production of follistatin and PDGF-BB (Yano, et al. 2006). This observation makes the significant difference between the groups in these analytes even more compelling.

The present study has shown the disrupted and sensitive response of cell-mediated cytokine and angiogenesis related protein secretion in malignant glioma patients. It has also shown that there are a specific set of proteins that react to tumour removal.

The luminex technique used is a reliable and reproducible technique that measures concentrations of multiple analytes simultaneously within four hours using only 50µl of sample and is clearly suitable for use in clinical practice.

4.4 Immunohistochemistry – Follistatin

Some gliomas exhibited follistatin immunostaining of tumour cells and many appeared to express gemistocytic morphology. However, staining was not uniform throughout the tumour sample and some cells were patently immuno-negative, (Figure 3.6). Positive immunostaining was entirely cytoplasmic with no membrane or nuclear component and other tissue elements within the sections, including blood vessels, were completely negative (Figure 3.6 b). There were no specific features of the tumours or constituent cells which were evidently predictive of immunopositivity or to account for the significant variability between individual tumours. The non-cancerous (viz. normal) brain tissue, obtained from patient's with non-malignant pathologies, was uniformly negative throughout and there was no staining of either neurones or glial cells

(Figure 3.6 c). There was a distinct interstitial stain in the presence of negatively staining cells that followed the axonal tracts of the sections (figures 3.6 d and e). There was no specific axonal staining and some of the axonal tracts did not take up any stain. The non-cancerous brain axonal tracts were uniformly negative (figure 3.6 f). There was some specific cytoplasmic staining of some cells from the choroid plexus (figure 3.6 g). This may suggest that follistatin is being secreted into the CSF and warrants further detailed CSF analysis of anigiogenesis-associated proteins.

Vaquero, et al. (2000) reported that increased expression of angiogenic factors are a good predictor of transformation from low grade glioma to high grade. The imbalance between activin A levels and its inhibitor follistatin may influence the progression from low grade to high grade. Zhang, et al. (2010) reported increased expression of activin A whilst there was little difference in the expression of follistatin between low and high grade samples which suggest an inhibitory role for follistatin in the transformation from low to high grade glioma. The overwhelming up-regulation of activin A in high grade gliomas (Zhang et al. 2010) is not sufficiently neutralised by the mediocre up regulation of follistatin and therefore its inhibitory effect is marginal. This hypothesis is supported in this work considering the results just discussed.

4.5 Immunohistochemistry – Leptin

The leptin immunohistochemistry showed some unanticipated results (figure 3.7 and table 3.14). There was some small concentration of staining in the interstitial spaces of non-cancerous brain. This staining was not considered to be an experimentally-induced artefact as the staining was consistent with other positive sections. As glial cells and micro-vessels are the main sites of the ObR

receptor it is to be expected that in areas of tissue that contain a high proportion of these, leptin is present. Conversely there were also some areas of negativity in the high grade glioma sections. The heterogeneous nature of glioma could indicate a clone of non-malignant cells interspersed within the tumour or cells that have newly differentiated and have not yet started to produce leptin.

The present results have shown that leptin was expressed at a low concentration (+) in the interstitial space of non-cancerous tissue but at higher concentrations in both the interstitial space and the cytoplasm in both low and high grade gliomas. Leptin was rarely located in the nucleus in both non-cancerous and glioma specimens, only producing a (+) stain in the highly malignant gliomas. These data are in contrast to those of Morash, et al. (2000) who localised leptin to the nucleus of C6 cells. Morash, et al. (2000) further stated that the data collected in their study suggested the presence of nuclear binding of leptin and that RT-PCR analysis using primers specific for OBRs (short form) and OBRb (long form) indicated that C6 cells can only express the short leptin receptor isoform.

These results show that the greatest staining of leptin by tumour cells is in the cytoplasm and that for each of the analysed areas (nucleus, interstitial space and cytoplasm) the staining intensity of leptin increased in correlation to the degree of malignancy.

4.6 Immunohistochemistry – Prolactin

The mediocre immuno-histochemical staining of prolactin in this study does not distinguish between endogenous, extra-pituitary prolactin and prolactin captured by prolactin receptors and tumour resection of the source of

production of the increased prolactin, (figure 3.8). These results are insufficient as an explanation for the post-operative decrease.

The studies of Soares et al. (2007) have supported the finding of increased serum prolactin. However the present study demonstrated 86% hyperprolactinaemia in glioblastoma patients and 75% in low grade glioma patients. These data must be examined in the light of the glioma patient's treatment regime. Other researchers have reported that anti-epileptic drugs may modulate hormone release from the hypothalamic-pituitary-gonadal axis and they may alter the metabolism of sex hormones and their binding proteins (Verrotti, et al. 2009). Clearly a patient's full drug history, not currently available, must be accounted for when reviewing these results but the abrupt decrease within twenty-four hours of tumour de-bulking suggests a tumour-based influence upon prolactin production.

4.7 Immunohistochemistry – PDGF

Aberrant PDGF signaling is known to play an important biochemical and physiological role in glioma cell transformation from multipotent neural cell progenitors (Calzolari and Malatesta 2010). Increased PDGFRα expression is linked with a loss of heterozygosity on chromosome 17p in the region of the tumour suppressor gene TP53 which signals a possible effect on PDGFRα expression (Hermanson, et al. 1996). Hermanson et al. (1996) further suggested that over-expression of PDGFRα could contribute to tumour cell proliferation in the early and late stages of glioma development. Staining of low grade biopsies for the PDGFRα moiety should be undertaken to confirm this.

(Martinho, et al. 2009) reported increased expression of PDGF-AA in 81.2% of 160 glioma biopsies. However, this does not confirm a correlation with PDGFRα

expression in contrast to this study in which the correlation was admittedly weak (r=0.5). There is sufficient support, however, for significant over-expression of the PDGF-AA ligand in glioma stem cells (Zhang, et al. 2015).

Semenza (2013) reported that hypoxic conditions could induce the production of hypoxia-inducible factors (HIFs) of which PDGF-BB is one. However (Costa, et al. 2012) demonstrated a role for PDGF-BB in the downregulation of microRNAs miR-21 and miR128 which was associated with increased cell proliferation. The increased expression of PDGFRα, PDGF-AA and PDGF-BB in this study, (figure 3.9 and table 3.15), suggests an autocrine and paracrine function for these molecules in cellular proliferation and tumourigenesis.

The decreased expression of PDGFRβ is in contrast to previous studies that noted an increased expression (Li and Xu 2016b). However, Brennan, et al. (2009) have postulated a link between an increase in PDGFRα expression and a decrease in PDGFRβ. This is supported by the data of the present study as there was only a single patient sample with an increased PDGFRβ expression and this was matched with a further increased PDGFRα expression. There was only a single staining of a non-cancerous control for PDGFRβ and further staining of non-cancerous samples would be beneficial in confirming the under expression in high grade glioma.

PDGFRα and PDGF-AA ligand expression displayed a significant positive correlation (r=0.5), (table 3.15). The present results could suggest that increased expression of PDGFRα could upregulate expression of the PDGF-AA ligand or vice-versa. If this is the case then an autocrine role in the promotion of cellular proliferation should be considered for these molecules (Venugopal, Wang et al. 2012). They showed that neural precursor cells exposed to GBM conditioned

media become highly proliferative and display an increased expression of growth factor receptors such as PDGFRα. This was augmented by an increased level of PDGF-AA in the GBM conditioned media. Further investigation into a possible autocrine activity for PDGFRα and PDGF-AA is warranted including an investigation of which molecule stimulates the upregulation of the other.

In addition, the present results show a significant correlation between PDGF-AA serum concentration (pg/ml) and PDGF- BB ligand expression in Primary GBM (r=0.89). Shure, et al. (1992) show that PDGF AA is a strong chemoattractant for human monocytes, granulocytes, and fibroblasts. Therefore, PDGF-AA elevation in glioma, can be attributed to the altered glioma immune environment. Interestingly, Zheng, et al. (2016) reported the link between the overexpression of PDGF-BB in glioblastoma and the recruitment of oligodendrocyte precursors expressing increased levels of PDGFRα which in turn lead to increased expression of PDGF-AA.

The interactions of the PDGF family of molecules in driving glioma cell proliferation is a complex and multi-layered process that may include several redundant and pleiotropic mechanisms. There are many methodological limitations that can compromise these results.

This work aimed to locate the source of PDGF receptor and ligand expression in high grade glioma and to examine any possible correlation with systemic concentrations. Of 18 high grade tissue sections PDGFRα had increased expression in 93.75% of specimens, PDGF-BB was increased in 75% and PDGF-AA in 43.75%. PDGFRβ had 25% of specimens showing increased expression and 56.25% decreased

expression. All sections were compared to non-cancerous control specimens.

These results suggest that PDGFRα/PDGF-AA signaling could lead to increased angiogenesis in addition to that due to oxygen requirement from hypoxic tissue. This stimulus would lead to an induction of hypoxia-dependent production of PDGF-BB which leads to an increased chemotactic response of peripheral immune cells which, in turn, contributes to an immunosuppressive micro-environment.

Immunohistochemistry does not reveal the source of the proteins only the location within the tissue therefore speculation on biological function is compromised. Staining intensity scoring is subjective and the ELISA method of serum analysis has low sensitivity. There was also a limited sample size of 6 patients due to availability of matched tissue and serum samples in which the correlations were calculated. A larger cohort would provide greater accuracy in this comparison.

4.8 Discussion of potential biomarkers

The platform of the serum analysis was based on a luminex technology and was performed on the Bio-Plex 200 analyser. This platform utilises many micro-beads coated with antibody of the analytes tested. The technique includes many washing steps for which a wash station was used. This automated method is preferred over the manual washing method and increases both reliability and reproducibility. The inherent variation induced by the operator's technique is thus minimised. The automated cell washer was validated before the assays were performed according to the manufacturer's instructions. (Bio-rad 2013) The mean percentage recovery of the beads was consistent with the

manufacturer's guidelines at 91% recovery. The process of testing the analytes takes somewhere around 4 hours with this technology. This could be improved in a clinical laboratory setting with a more automated version of the luminex analyser. Other potential methodologies to measure the analytes include mass spectrometry and chip technology. Both of these methods are considerably more expensive than the luminex technique used in this study. There was no correlation with any of the potential biomarkers and survival or gender, (table 3.21). This was not surprising due to the heterogeneity of the tumour and the many factors influencing the production of the analytes in question. A more detailed longitudinal study of the analytes from patients could provide more prognostic information for instance the likelihood of tumour recurrence following removal.

The increased levels of some analytes in the CSF mirrored those within the serum (figures 3.10 and 3.12). This is an interesting finding that warrants further investigation although it should be noted that CSF is a more intrusive fluid to obtain for diagnosis compared with blood serum.

Prolactin has been traditionally associated with mammary development and lactation although, more recently, several studies have implicated the hormone in tumourigenesis through disruption of the JAK-STAT pathway (Gorvin 2015). These studies are restricted to breast cancer, however the role of prolactin and its receptor in glioblastoma is being elucidated. Other studies have detected the presence of intracellular prolactin, prolactin receptor (PRL-R) and hyperprolactinemia in different types of central nervous system (CNS) tumours. Ciccarelli, et al. 2001; Van Meir et al. 1990 discovered the presence of PRL-R and hyperprolactinemia in 45.4% and 27.2% of meningiomas and 69.2% and

61.5% of schwannomas respectively. In these studies the level of the receptor and the ligand were not correlated. Soares et al. (2007) discovered the presence of PRL-R in 39% of patients with CNS tumours and the presence of intracellular prolactin in 21.9% of those patients. There was also hyperprolactinaemia in 30% of 82 cases. Their data suggested a positive correlation with serum prolactin and intracellular prolactin. Both of these studies confirm the data obtained in the present study of increased serum prolactin however the present study demonstrated 86% hyperprolactinaemia in glioblastoma patients and 75% in low grade glioma patients.

These data must be examined in the light of the glioma patient's treatment regime. Some researchers have reported that anti-epileptic drugs may modulate hormone release from the hypothalamic-pituitary-gonadal axis and they may alter the metabolism of sex hormones and their binding proteins (Verrotti, D'Egidio et al. 2009). Clearly, a patient's full drug history must be accounted for when reviewing these results but the abrupt decrease within twenty-four hours of tumour de-bulking suggests a tumour-based influence upon prolactin production. The mediocre immuno-histochemical staining of prolactin in high grade tissue samples discussed in chapter 4 does not distinguish between endogenous, extra-pituitary prolactin and prolactin captured by prolactin receptors and tumour resection of the source of production of the increased prolactin is insufficient as an explanation for the post-operative decrease.

Granulocyte colony stimulating factor (G-CSF) is a cytokine in routine use in cancer treatment which enables haemopoietic rescue following chemotherapy and radiotherapy. This cytokine also has a dual role in stimulating both immune-

suppression and tumour mediated angiogenesis in glioma (Aliper, et al. 2014 ;Wang, et al. 2012). This has resulted in the limitation of its use to prevent the iatrogenic induction of tumour growth.

Soluble human epidermal growth factor receptor 2 (sHER2-neu) is a circulating biomarker using in monitoring treatment efficacy in breast cancer (Stieber, et al. 2011). (Kostler, Steger et al. 2004) reported that breast cancer patients with decreasing sHER2-neu levels 30 days post-treatment with trastuzumab and chemotherapy showed a greater response to the treatment given. The decreased sHER2-neu levels in the glioma patients, shown in the present study, may be a reflection of the treatment regime of the patients in the study. Whilst the treatment for glioma does not intentionally target sHER2-neu, this could be an effect of the chemotherapy given to the high grade glioma patients.

Interleukin-6-mediated activation of Stat3 is a principal pathway controlling tumourigenesis (Sansone and Bromberg 2012). This transcription factor has a role in the regulation of specific mediators of tumour progression and is a promising therapeutic target (Roxburgh and McMillan 2016). The present study shows an increase in the soluble IL-6 receptor in high grade gliomas which may be a reflection of the increased mediation of the STAT pathway in tumour cell propagation.

The components of the plasminogen-plasmin system of fibrinolysis have been associated with increased cell migration and proliferation. These include uPA, uPAR and their inhibitor PAI-1 and (McMahon and Kwaan 2015) have noted utility in the prognostic information received with regard to numerous cancers including glioma. uPA is also the subject of a targeted therapy with authors

reporting inhibition of glioma cell migration through inhibition of the uPA molecule itself (Chou, et al. 2015). With the significant increase of this marker noted in this study, there is a pattern emerging of an angiogenetic panel of markers within the field of potential diagnostic biomarkers for glioma.

The distinction between high and low grade gliomas relies on the fact that high grade gliomas are malignant and invasive and therefore have a higher rate of angiogenesis. Several of the potential biomarkers have been associated with increased cell migration and proliferation of glioma cells *in vitro* (Becker, et al. 2010;Lee, et al. 2014). Anti-angiogenic therapies are being explored as treatment strategies in clinical trials. Chae, et al. (2010), demonstrated a complex interaction of expression of some of the potential biomarkers (sVEGFR-1) in response to drug therapies and a detailed history of the participant's treatment regime may be necessary to fully elucidate the expression pattern of the potential biomarkers in the individual patient. More recent trials have been unsuccessful however (Chowdhary and Chamberlain, 2013).

Hypoxia-induced angiogenesis is one of the key models for the cause of microvasculature with GBM. Tumour cells that outgrow endogenous blood supply undergo hypoxia, mediated by HIF-1α transcription, leading to the transcription of angiogenic factors, vascular endothelial growth factor (VEGF-A), platelet-derived growth factor ligand BB (PDGF-BB) and follistatin, (Charles, et al. 2012b). The tumour therefore mediates its own progression through healthy tissue in creating the angiogenic factors neccessary for the formation of new blood vessels. As surgery reduces the hypoxic area of the tumour, the stimulus for such production is also reduced. Therefore a further potential cause

for the drop in Follistatin concentration in postoperative patients could be the reduced hypoxic drive of the tumour following debulking surgery.

The platelet-derived growth factor receptor beta (PDGFRβ) has been noted on tumour vasculature cells (Li and Xu 2016b) and as PDGF-BB can signal through all PDGF receptors. It is a promising ligand to drive tumour progression, and has been noted to upregulate the production of VEGF-A – an important angiogenic factor (Xue, et al. 2013). The present study measured serum levels of PDGF AB BB only and a clearer elucidation of the different individual isoforms of PDGF (AA, AB and BB) and their receptors is required to resolve the contribution of these to the increase in serum levels of PDGF and VEGF-A.

The role of cell adhesion molecules in glioma angiogenesis and invasion has also been documented (Burim, et al. 2009). Whilst the present study included PECAM-1 only, the work of Burim et al. (2009) suggests that polymorphic variants of ICAM-1 are correlated with diffuse Grade II astrocytomas.

VEGF-A is a key mediator of angiogenesis, increasing in serum levels proportionally to the vascularity of gliomas, and complexes with sVEGFR-1 reducing its bioavailablity (Lamszus, et al. 2003). Lamszus et al. (2003) suggest that whilst sVEGFR-1 is increased in glioblastoma, in agreement with the present study, the sVEGFR-1: VEGF-A ratio is decreased in these more malignant gliomas thereby increasing the bioavailability of VEGF-A and hence an increase in angiogenesis. The usefulness of sVEGFR-1 as a potential biomarker would therefore be linked to VEGF-A levels in an individual patient.

The tumour microenvironment is pro-angiogenic and it is composed of cancer cells, stromal cells and immune cells including cancer-associated fibroblasts.

Interestingly (Katoh 2016) reported an increased efficacy of fibroblast growth factor (FGF) inhibition when combined with VEGF inhibition in producing anti-tumour effects in cultured cells. Serum FGF levels have been shown to be elevated in both oesophageal and lung cancers (Keeley, et al. 2014). Keeley et al. (2014) reported that FGF levels were elevated up to two years before diagnosis of such cancers.

There is a convincing correlation between the levels of potential biomarkers in this study with increased levels of malignancy and therefore angiogenesis (Pang, et al. 2012; Majumdar, et al. 2009).

The significant changes of three potential biomarkers, follistatin, G-CSF and prolactin after de-bulking, (figure 3.13), led to Lekka et al. (2015) suggesting that these have potential for use in monitoring patient treatment. These data support the hypothesis of the tumour being the source of some of the potential biomarkers.

4.9 Discussion of novel diagnostic testing strategy

The data obtained in the present study suggest that panels of multiple biomarkers should be considered for diagnostic use and an orthogonal testing regime be formulated (table 4.1). The potential biomarkers would need to undergo rigorous testing in the confirmation, assay development and validation stages of biomarker approval before a reference range could be produced.

The first panel should consist of those biomarkers with the greatest power to distinguish tumour from non-cancerous serum as well as the ones that show a

decrease upon tumour removal. A secondary panel of biomarkers should then be tested to distinguish grade of tumour. (Table 4.1a and 4.1b).

Table 4.1. A prospective diagnostic testing pathway to distinguish between low grade and high grade glioma from non-cancerous serum.

a)

Panel 1	Expected result for HGG sera	Expected result for LGG sera	Expected result for non-cancerous sera
Prolactin	Increased	Increased	Normal range
sVEGFr-1	Increased	Increased	Normal range
FGF-basic	Increased	Increased	Normal range
Follistatin	Increased	Increased	Normal range
G-CSF	Increased	Increased	Normal range
sHER2-Neu	Decreased	Decreased	Normal range

b)

Panel 2	Expected result for HGG sera	Expected result for LGG sera	Expected result for non-cancerous sera
sIL-6R alpha	Increased	Normal range	Normal range
uPA	Decreased	Normal range	Normal range

The studies performed have shown differences in the means of the glioma and non-cancerous samples. To generate a putative cut-off point the following procedure was used. A 95% confidence interval was used to calculate a normal reference range with the non-cancerous sera. An increase or decrease of 2 SD in the cancerous cohort was used to calculate a positive diagnostic level of the biomarker. The average number of positive biomarkers in the 115 high grade patients was 3.79, for the 44 low grade patients it was 3.56. The non-cancerous

patients were falsely positive in 1.56 biomarkers. The sensitivities and specificities of using different numbers of positive biomarkers to confirm diagnosis in panel 1 is given in table 4.2. These results were calculated by looking at each individual glioma patient and assessing how many of the stated biomarkers they were positive for. Therefore, 100% of high and low grade glioma patients were positive in at least one of the biomarkers giving a sensitivity of 100% however 75% of the non-cancerous cohort were also positive in one of the biomarkers hence there is a specificity of 25% using only one biomarker. The optimum number of positive biomarkers in panel 1 to aid diagnosis would be 2 although using 3 would significantly reduce the numbers of false positives by 12%.

Table 4.2. Sensitivity and specificity of panel 1 compared with the number of positive biomarkers used.

No. of positive biomarkers	Sensitivity for high grade	Sensitivity for low grade	Specificity
1	100%	100%	25%
2	94%	89%	64%
3	75%	73%	76%
4	56%	55%	85%
5	43%	30%	94%
6	11%	7%	100%

Table 4.3 shows the sensitivities and specificities for using 1 or 2 biomarkers to distinguish between low and high grade glioma. There is a trade-off between sensitivity and specificity depending on the number of biomarkers used. Discovery of more biomarkers to distinguish between the grades would allow for greater resolution of glioma grade using panel 2. The differences in serum

concentration between cancerous and non-cancerous sera for sIL-6R alpha and uPA given in figure 3.10 were significant albeit only at the $p<0.05$ level. Therefore this lower significance level contributes to the resultant lower sensitivities found in panel 2.

Table 4.3. The sensitivity and specificity of panel 2 compared with the number of positive biomarkers used.

No. of positive biomarkers	Sensitivity for high grade	Sensitivity for low grade	Specificity for high grade
1	90%	84%	27%
2	38%	27%	85%

4.10 Scope for future studies

The cryopreservation of samples presented some technical problems that may have had an adverse impact on the accuracy of the results obtained. Future experiments should use fresh blood and a density gradient medium to separate peripheral blood mononuclear cells (PBMCs) as suggested by other researchers to reduce the non-specific staining causing autofluorescence (Mallone, et al. 2011). A more accurate method of assessing cellular viability using propidium Iodide in a flow cytometric method rather than the manual method of the trypan blue assay would have been useful although both trypan blue and propidium iodide assays are limited as these assays only represent the intactness of the cells and have little relation to the functionality of the cells.

The effect of cryopreservation would be better assessed by the testing of fresh samples and then re-testing the samples after specific periods of time to see if there is any decrease in viability and a change in the production of TNF-α.

The present study took no account of circadian rhythms of cytokine secretion and a log of the time of day the sample was taken was not performed. Likewise there was no data relating to patient age and gender taken with the samples and so any effect from these factors was ignored. Further study should be done to evaluate the correlation of other demographic metrics such as age, drug therapy and gender with TNF-α.

The experiment that compared the presence of Tregs between glioma and meningioma would have been better served by including a non-cancerous cohort. The low serum levels of Treg cells in malignant and non-malignant tumours may be due to the fact that Tregs migrate towards the site of the tumour and be embedded within the tissue. Further work should be performed to find evidence of tumour infiltration of the Treg compartment as well as comparing serum levels between tumour and non-cancerous patients.

Lastly, TNF-α was chosen as the cytokine to measure in this experiment due to its influence on the induction of apoptosis with higher levels indicating an increased number of cells undergoing apoptosis. However the induction of apoptosis is a complex process involving other receptors and the production of TNF-α by other cell types such as macrophages, monocytes and fibrinoblasts (Bao and Cao 2014). The present study provides only a crude snapshot of the process which may explain the lack of difference in most of the patient levels following cryopreservation and further research into the role of TNF-α as an apoptotic factor in lymphocytes would be beneficial.

The immunohistochemical staining of leptin revealed the localisation of leptin did not occur in the nucleus. However Morash, et al. (2000) revealed different

forms of the leptin receptor in C6 cell lines and other researchers demonstrate that only the short form receptor has leptin-dependent signalling potential (Bjorbaek, et al. 1997). Its presence suggests an autocrine function for leptin in glioma tissue. As the present study used primary tissue RT-PCR analysis of primary glioma tissue should be investigated to see if the results of Morash et. al (2000) in a glioma cell line can be replicated in primary tissue and therefore the production of leptin in glioma patients can be re-evaluated.

The distribution of the OBr receptor may influence the localisation of the leptin ligand and so future studies should indicate the precise location and amount of leptin receptors in non-malignant tissue to enable a more meaningful comparison with leptin distribution in malignant tissue.

Likewise, the inability of the prolactin staining to distinguish between endogenous secretion and prolactin captured by prolactin receptors prevents an informed discussion of the stark decrease in serum levels following resection. Further investigation of the presence of the prolactin receptor (PRLR) in both non-cancerous and glioma tissue would demonstrate whether the receptor is saturated with extra-pituitary prolactin ligand in glioma patients. This study should be corroborated by mRNA extraction coding for prolactin to elucidate further the source of the increased serum levels.

The immunohistochemical analysis of PDGF ligands and receptors revealed a complex interaction between PDGFRα and PDGF-AA signalling that may lead to increased angiogenesis and a hypoxia-dependent production of PDGF-BB. Future study should look at the molecular interactions of the PDGF family of

ligands and receptors with regards to a therapeutic strategy of disruption or blocking of these interactions to prevent or reduce angiogenesis.

Some cancer-specific treatment modalities warrant the use of haemopoietic rescue following a chemotherapeutic regime using G-CSF as a stimulator of haemopoietic stem cells. Clearly, the increase in serum levels of G-CSF shown in this study suggests that an investigation into the precise role of G-CSF in glioma production is required in order to determine treatment efficacy as well as to contribute to unravelling the source of the post-operative reduction in G-CSF levels. Another potentially artificially-induced analyte increase is in the soluble IL-6 receptor. Whether this is tumour-produced or as a result of an inflammatory response to the malignancy is a subject of further investigation involving a non-cancerous patient cohort with an inflammatory condition.

Some of the candidate biomarkers discussed in this study are being considered as potential markers for other potential targets. Some of these are polymorphic variants of the markers already discussed that may be more responsive to glioma progression. ICAM-1 is a further candidate for a potential biomarker to investigate with regard to low-grade glioma progression to more malignant grades. Other biomarkers such as VEGF A and its interaction with its receptor display a dynamic change during glioma progression. Further study is required to determine the dynamics of the sVEGFR-1: VEGF-A ratio from progression towards malignant forms of glioma. This ratio may be a more specific and therefore more revealing metric in disease monitoring. Also a historical perspective may also be necessary as FGF levels may be artificially increased a considerable time before the earliest diagnosis. The present study suggests an elevation in the serum levels of FGF in glioma patients however there may

be evidence to warrant an investigation into pre-diagnosis samples if they could be obtained.

Angiogenesis, as one of the hallmarks of cancer, leaves a footprint with the potential biomarkers in this study however the scale of correlation with the amount of angiogenesis taking place remains unknown. Measurements of vascularisation in individual patient's tumour sections should be undertaken to investigate any possible correlation with serum levels of the biomarkers.

Many of the significant serum analytes also displayed an increase in CSF samples albeit from only 8 samples available suggesting that the source of production of some of the potential biomarkers has access to both the bloodstream and CSF. This points towards the tumour itself as the site of production of the analyte although more samples would be needed to verify this in a future study. Future work would include a longitudinal study.

In this future study there will be an investigation of a comprehensive panel of blood-borne biomarkers that potentially may have predictive or prognostic significance in a large series of glioblastoma patients recruited from the two major treatment centres in the North West. In addition longitudinal biomarker studies will be undertaken in patients treated with combined temozolomide and radiotherapy to assess the relationship of markers with tumour burden, response to therapy and progression. The prognostic significance of T lymphocyte populations will also be assessed.

Adult patients of age greater than18 years, will be recruited prospectively at the Walton Centre / Clatterbridge Centre for Oncology and the Royal Preston Hospital and all patients with glioblastoma who give consent for research will

be included in the study irrespective of their performance status and clinical management. The aim is to recruit 250 patients overall. Patients with newly diagnosed glioblastomas as well as those with an earlier lower grade tumour will be included. Longitudinal investigations of biomarkers in comparison to imaging and outcome will be undertaken for good performance patients treated with concurrent and adjuvant temozolomide and radiotherapy. Based on current clinical practice approximately 80 patients would be available for this part of the study.

Blood samples will be obtained at initial diagnosis pre-surgery for all patients and at defined stages during the clinical: - at initial diagnosis, 24 hours post-surgery, pre-therapy; after radiotherapy [prior to adjuvant therapy]; after adjuvant therapy; at 3 months then 6 monthly post therapy; at recurrence or suspected recurrence, and at intervals during further therapy and follow up as long as practical depending on the individual patient. Blood samples will be taken as close as possible to neuroradiology investigations. Tumour tissue samples from further surgeries would be stored for comparative studies. Blood samples will be collected and processed according to a standard protocol used in both hospitals to generate serum, plasma and cellular fractions.

Tumours will be diagnosed according to WHO criteria. For research purposes, independent histopathology re-review will be undertaken by the consultant neuropathologists at each hospital and features such as histopathological phenotype, lymphocyte and T- cell infiltration, characteristics of the vasculature, extent of apoptosis and necrosis will be assessed.

Clinical data should be collated prospectively and will include details of therapy (dose, times), documented progression date and whether radiological, clinical or both, steroid doses at the intervals when blood samples are taken, progression free survival, details of further surgery or therapy and overall survival. MR and CT scans taken for diagnosis and at all stages of clinical management will be compared with biomarkers and volumetric assessment used to assess the relationship between markers and tumour burden. Scans will be used to monitor therapy response or disease progression or suspected pseudo progression.

Protein biomarkers present in serum or plasma will be measured and pre-surgery, pre-therapy levels in glioblastoma patients (n=100) compared with normal healthy volunteers (n=50) and gliomas WHO grades II (n=50) and III (n=30). For markers significantly deregulated in glioblastoma, comparison of expression in different molecular subtypes of glioblastoma will be made with outcome and with expression levels in tumour tissue. Biomarkers in longitudinal samples from patients treated with temozolomide and radiotherapy will be compared with therapeutic response assessed using the 'response assessment in neuro-oncology' (RANO) criteria used clinically for measuring a patient's response to radiotherapy, and volumetric measures of tumour size using T1+ gadolinium and T2 MR at discrete stages following therapy and at recurrence.

Diagnostic archival samples and snap frozen tumour tissues taken as part of planned surgery and surplus to diagnostic requirements will be available for each case for molecular classification (MGMT methylation and IDH1 mutation

status) and for comparison of biomarkers in blood with expression in tumour tissues.

4.11 Conclusion

A biomarker with high sensitivity and specificity to aid glioma diagnosis would be of great clinical utility. Current methods of diagnosis are invasive with the diagnostic test requiring hospitalisation. A rapid test based upon blood serum and would allow for a relatively non-invasive test and open up the possibility of screening for glioma as well as effective treatment monitoring and prediction of tumour recurrence. Analysis of the source of the proteins may reveal information regarding the mechanism of immunosuppression in these tumours. The present work has demonstrated the ability and potential of specific biomarkers to aid diagnosis of glioma in a patient's blood sample. The diagnosis can be rapidly applied to the clinical environment allowing the possibility of screening of patients with equivocal symptoms of glioma upon first presentation which would be of great benefit to the clinician in improving patient outcome.

This work studied both high and low grade glioma patients using serum, buffy coat and tissue samples supplied from the Brain Tumour North West research cluster under their ethical procedures. The studies were age and sex matched as much as possible. The main finding from these studies were as follows;

General anaesthetic does not affect the levels of biomarkers therefore the differences are most probably disease-based.

Tumour resection reduces some biomarker levels, suggesting a tumour cell source of some of the potential biomarkers.

Diagnostic information cannot be based on a single biomarker but a panel of biomarkers can be developed to distinguish between both high and low grade tumour patients and non-canceropus controls.

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Appendix A

Analytes	female mean pg/ml / sd	male mean pg/ml /sd	n female	n male
Prolactin	28707 / 43389	21159 / 17091	88	133
sVEGFr-1	867.5 / 309	922.4 / 329	88	133
FGF - basic	206.9 / 60	214.6 / 60	88	133
Follistatin	1847.4 / 700	1934.5 / 736	88	133
G-CSF	468.6 / 417	497.7 / 473	88	133
sHER2-Neu	3607.5 / 1545	3881.5 / 1524	88	133
sIL-6R alpha	10732 / 5172	11297 / 5112	88	133
PECAM-1	4805 / 1445	4914 / 1480	88	133
uPA	232.7 / 63.2	253.7 / 87.8	88	133
PDGF-ABBB	7789.5 / 3158	8213.7 / 3537	88	133