MALIGNANT GLIOMA
CHEMOSENSITIVITY TESTING AND BIOMARKERS

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF MEDICINE (M.D)

University of Central Lancashire

2012

In Collaboration with Lancashire Teaching Hospitals NHS Trust

DECLARATION

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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ABSTRACT

Chemosensitivity testing and molecular biomarkers are commonly used methods during the management of several types of malignant tumours in routine clinical practice. The present work attempts to transfer the use of these two concepts to the management of malignant gliomas.

Chemosensitivity testing was performed on twelve primary malignant glioma cultures at passage 0. The drugs tested were Temozolomide, Cisplatin and Carmustine, which are commonly administered chemotherapeutic agents in clinical practice in our institution. The results revealed that cultures derived from females or from patients under 65 years of age were more sensitive to the drugs used. More importantly, the chemosensitivity *in vitro* results were predictive of the clinical outcome of the donor patient. During chemosensitivity testing, Temozolomide was found to have poor *in vitro* effect. Increased frequency of application as well as cell cycle synchronisation of glioma cell lines were found to improve the kill efficiency of Temozolomide.

Six molecules were considered as potential biomarkers. All of them were found to be effective at predicting the presence of malignant gliomas. With the exception of VEGF and PDGF-BB, this is the first report of Follistatin, IL-6, IL-8 and IL-10 as biomarkers for malignant gliomas. Overall age and gender of patients did not affect the biomarker results.

Brain tumours represent 2% of all cancers in the UK. Early detection and treatment of glioblastoma is challenging and little progress has been made so far. This imposes a clear need for improving the diagnosis and management of malignant gliomas. This study aims to address both challenges and proposed ways of improvement.

Acknowledgements

I am deeply indebted to both my supervisors, Prof Tim Dawson and Prof Bob Lea for their support and enthusiasm during these three years. They were both very approachable and instrumental in guiding me through this project. Mr Gregory Hall was always easily approachable and available to give directions when I needed help.

I could not have been luckier in having Kate Ashton as laboratory manager. Her competence, ability and help were second to none. She did not only assist me in all my experiments but also saved me time with her organisation and record keeping. Julie Shorrocks has been the vital link between the two laboratories I used for this project. Her help, guidance and availability are greatly appreciated.

Prof Charles Davis has inspired me into becoming a Neurosurgeon with Neuro-Oncology interest. His constant encouragement and moral support kept me focused during some difficult times.

I would also like to thank all my colleagues in the Preston Neurosurgical Unit, who are too numerous to mention, but without their help it would have been impossible for me to carry out this study.

I would also like to thank all the patients and relatives who have generously participated in this study while going through a very difficult time in their lives. Without their generosity not only the current project, but none of the previous ones undertaken in the Preston Neurosurgical Unit would have been possible.

Lastly but not least, I would like to thank my husband Nicolas. Without his help, knowledge, skills, encouragement, understanding, guidance, patience and unconditional love, this work would not have been finished.

Table of Abbreviations

Abbreviation	**Expansion**
MDM2	Mouse Double Minute 2
PTEN	phosphatase and tensin homolog deleted on chromosome 10
EGFR	Epidermal Growth Factor Receptor
PDGF	Platelet Growth Factor
PDGFR	Platelet Growth Factor Receptor
BRU	Bromodeoxyuridine
DNA	Deoxyribonucleic acid
GFAP	Glial fibrillary acidic protein
RNA	Ribonucleic acid
WHO	World Health Organisation
CNS	Central Nervous System
SSEA-1/Lex	Stage specific Embryonic Antigen/Lewis X
Rb	Retinoblastoma
CDK	Cyclin Dependent Kinase
EGF	Epidermal Growth Factor
VEGF	Vascular Endothelial Growth Factor
TGF	Transforming Growth Factor
FGF	Fibroblast Growth Factor
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
CT	Computerised Tomography
MR	Magnetic Resonance
MRS	MR Spectroscopy
fMRI	Functional MRI
PET	Positron Emission Tomography
DTI	Diffusion Tensor Imaging
DSC-MRI	Dynamic susceptibility-weighted contrast-enhanced

	perfusion MR
BBB	Blood Brain Barrier
MAP4	Microtubule Associated Protein 4
BCNU	Carmustine
CCNU	Lomustine
MGMT	O6-MethylGuanine-Methyl-Transferase
IFN	Interferon
IL	Interleukin
TNF	Tumour Necrosis Factor
HSV-tk	Herpes Simplex Virus Thymidine Kinase
MMPI	Matrix Metalloproteinase Inhibitors
MMP	Matrix Metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
TMZ	Temozolomide
DMEM	Dulbecco's Modified Eagle's Medium
HBSS	Hank's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
Cis	Cisplatin
PBS	Phosphate Buffered Saline
LD	Lethal Dose
KS test	Kolmogorov-Smirnov statistical test
GCS	Glasgow Coma Scale
BTNW	Brain Tumour North West
CSF	Cerebro Spinal Fluid
NCIC	National Cancer Institute of Canada
MTIC	5-(3-methyl triazen-1-yl) imidazole-4-carboxamide
WBC	White Blood Count
ECG	Electro Cardio Gram
CRP	C Reactive Protein
BUN	Blood Urea Nitrogen
TG	Triglyceride

GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
LFT	Liver Function Tests
U+E	Urea and Electolytes
AED	Anti Epileptic Drugs
PD	Parkinson's Disease
MS	Multiple Sclerosis
EC	Endothelial Cells
HGF	Hepatocyte Growth Factor
PGE	Prostaglandins
PF4	Platelet Factor 4
TSP-1	Thrombospondin-1
FLT	Follistatin
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule-1

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1 GENERAL INTRODUCTION

1.1 Glioma incidence and epidemiology

Gliomas are the commonest primary brain tumours accounting for almost 2% of all newly diagnosed malignancies every year in the UK [1]. Amongst the gliomas, malignant gliomas represent approximately 60% of the primary brain tumours, making them the commonest histological type of primary brain tumours diagnosed every year. Their incidence almost compares with the yearly incidence of leukaemia, being 7 per 100,000 population. They account for 7% of the years of life lost from cancer before the age of 70 [1]. The incidence for males is 8.8 and females 6.2 per 100,000 population. There were 3674 deaths occurring from brain tumour malignancies in the UK in 2008, with the peak mortality during the 7th decade of life for both sexes. The overall 1-year survival of patients with all histological types of brain malignancies is approximately 50%, but drops to 23% at 5 years. The 5-year survival of young adults (age 15-39 years) is 32%, nine times the one of the older population (age 60-79 years) [1].

The incidence of malignant gliomas varies by age, with a gross difference between childhood and adulthood. In childhood, malignant brain tumours represent the commonest solid tumour. The incidence peak drops in teenage years and steadily increases to a second peak in the 7th decade of life [2].

Tumour location is also different between these age groups. Malignant gliomas in the childhood are most often found in the infratentorial compartment or in the midline, while in the adult population, the commonest site of these malignancies is the supratentorial compartment.

1.2 Glioma classification

Gliomas are classified according to the phenotype of the cell type they mimic as astrocytoma, oligodendroglioma and ependymoma. The cell of origin of the gliomas on the other hand, remains uncertain. The theory that seems to be gaining acceptance is the cancer stem cell theory. Stem cells have the ability to divide and differentiate into diverse and specialised cell types. The two cardinal features that define stem cells are the ability to self renew and the totipotency. Self-renewal is the capacity that these cells have to divide while they maintain an undifferentiated state. There are two theories of the self-renewal mechanism. The first one is the obligatory asymmetric replication, where a stem cell divides into one daughter cell identical to the one of origin and another daughter cell that differentiates to a specialised cell. The second mechanism is the stochastic differentiation, where one stem cell develops into two differentiated daughter cells, while another stem cell divides into to stem cells identical to the parent cell.

The potency of these cells makes them able to differentiate into specialised cell types. Totipotent are stem cells able to differentiate into embryonic and extra embryonic cells, which are able to give rise to a complete organism. These cells are typically produced after the fusion of an egg to a sperm cell.

It is becoming increasingly evident that many, if not all, tumours originate from cancer stem cells. These cells are cancer cells that share common characteristics with normal stem cells, namely the ability to self-renew and to give rise to a particular cancer cell type. There is growing evidence that in the case of brain tumours, the cell of origin is a cancer stem cell [3].

Malignant gliomas grow rapidly, invade and destroy surrounding brain tissue but rarely metastasize. Classification is the major factor that clinicians take into account to decide on treatment. In Neuro-oncology histological classification is

an important factor in determining prognosis and the type of treatment offered to the patient. It is therefore imperative that the classification system of brain tumours is as accurate as possible. It is clear on the other hand that brain tumours that fall into the same histological type, respond very differently to the same treatment. It is to date impossible to distinguish between the responding and non-responding tumours just on the basis of the histological classification. This is one of the limitations of the current classification system. There is, though, a continuous re-evaluation of the existing classification of brain tumours, which is updated on a regular basis. The updates take into account advances into the molecular studies of gliomas in an attempt to classify the disease in a way, which will precisely guide therapy.

The most widely used classification of brain tumours is the World Health Organisation (WHO), which was revised in 2000. This classification differentiates malignant gliomas into four grades, according to the histological hallmarks present (see Table 1-1). Astrocytomas represent 25 – 30% of all adult gliomas.

Pilocytic astrocytomas are classified as Grade I tumours. They are common in children, located in the cerebellum and are characterized by the presence of Rosenthal fibers. Diffuse astrocytomas, or Grade II tumours, are characterized by the presence of hypercellularity with pleomorphism and nuclear atypia, while Grade III or anaplastic astrocytomas show mitotic activity and cellular anaplasia. Presence of vascular endothelial proliferation and or necrosis makes the tumour the highest grade, Grade IV also known as Glioblastoma (formally suffixed “Multiforme”). The Kernohan grading system of gliomas classifies the disease according to progressive malignancy, and groups Grade I and Grade II to make the Low Grade Glioma group and Grade III and Grade IV tumours to form the High Grade Gliomas. The prognosis, survival and treatment of these two groups is different [4]. Table 1-1 summarizes the WHO Grading of Astrocytomas.

Table 1-1 WHO Grading of Astrocytomas

WHO grade	Classification	Histological features
I	Pilocytic Astrocytoma	Rosenthal fibres + piloid cells
II	Diffuse Astrocytoma	Nuclear atypia
III	Anaplastic Astrocytoma	Cellular anaplasia and mitoses
IV	Glioblastoma multiforme	Anaplasia, mitoses, vascular endothelial proliferation , necrosis

Recent studies of molecular genetics have suggested that glioblastomas may arise by two separate pathways, primary or *de novo* glioblastoma and secondary glioblastoma. Secondary glioblastomas arise by malignant progression from a low grade glioma whereas *de novo* glioblastomas arise without a lower grade precursor. The two subtypes of glioblastoma are characterized by different genetic alterations. The *de novo* glioblastomas frequently have *EGFR* gene amplification, overexpression of *Mouse Double Minute 2 (MDM2)* and *PTEN* mutations whilst the secondary tumours show *TP53* gene mutations and *PDGFR-α* overexpression [5, 6]. The oligodendrogliomas as well as the ependymomas are less common and are classified according to three grades. Table 1-2 summarizes the grades and the histological hallmarks of each group [4].

Table 1-2 WHO Grading of other gliomas.

WHO grade	Classification of Oligodendroglial tumours	Histological features
I		
II	Oligodendroglioma	Round nuclei, fine reticular capillary network and microcalcification, perinuclear halo
III	Anaplastic Oligodendroglioma	Mitotic activity
WHO grade	**Classification of Mixed Gliomas**	**Histological features**
I		
II	Oligoastrocytoma	Variable cellularity, nuclear pleomorphism, limited mitotic activity
III	Anaplastic Oligoastrocytoma	Increased cellularity, nuclear pleomorphism, mitotic activity, microvascular proliferation
WHO grade	**Classification of Ependymal Tumours**	**Histological features**
I	Subependymoma	Low cellularity, low proliferation, isomorphic tumour cell nuclei
I	Myxopapillary Ependymoma	Perivascular rosettes, ependymal rosettes
II	Ependymoma	Perivascular pseudorosettes, mitotic activity, vascular proliferation
III	Anaplastic Ependymoma	Mitoses, perivascular pseudo rosettes, vascular proliferation, necrosis

1.3 Molecular Markers in Gliomas

Neuropathology has evolved in using several molecular markers, which highlight the tumour biology. These are useful in demonstrating the neoplastic changes at a molecular level; facilitate diagnosis as well as contribute to the prognosis of the tumour. The molecular markers used in neuropathology are

markers of proliferation, immunohistochemical markers and genetic markers [7].

Recently, there has been an increasing focus on molecular markers in malignant gliomas that may provide prognostic information or predict response to treatment. The goal of molecular genetics is to individualize patient treatment regimens and to allow monitoring of treatment response.

1.3.1 Markers of proliferation

Counting the mitotic figures in the tumour has been currently superseded by the Ki-67 and the bromodeoxyuridine (BRU) labelling indices, although BRU is not used frequently nowadays. The frequency of immunostaining for Ki-67 (a marker of cycling cells) or the frequency of the incorporation of the bromine analogue of deoxyuridine (a marker for cells in the S phase of the cell cycle) in tumour cell DNA, gives an estimate of cells that are actively dividing. This index generally correlates with increasingly malignant behaviour such as tumour necrosis, vascular proliferation, high invasiveness and dedifferentiation. These indexes are important in evaluation of tumour growth rate and clinical outcome and add a valuable tool in predicting outcome to tumour histopathology [8, 9].

1.3.2 Immunophenotypic markers

Immunohistochemistry is the process of detecting antigens in cells of a tissue section. It uses antibodies and their affinity to specific antigens found on the cell that requires identification. The antibody-antigen interaction is visualised by tagging the antibody to a fluorophore or to an enzyme such as peroxidase that catalyses a colour-producing reaction. Histochemical markers are proteins and glycolipids and are used to classify tumours by their cell type and degree of differentiation. Glial fibrillary acidic protein (GFAP) is a protein produced by normal astrocytes as well as by glial, intraepithelial and some ependymal tumours, and is widely used for this purpose.

Another type of immunohistochemical marker used currently is monoclonal antibodies against tumour specific antigens. They are used to study the pattern of expression of these antigens on the tumours. They may guide in the future selecting specific therapies for the tumours that express them [10].

1.3.3 Genetic markers

Genetic markers are small sequences of DNA specific to chromosomal locations and are associated with particular traits. They are usually used to trace inheritance of disease, guide diagnosis and treatment of disease. In neuropathology they are vital for the diagnosis as well as the classification of brain tumours. Examples of genetic aberrations are degree of aneuploidy, chromosome deletions, translocations and amplifications. Those may signal the loss of a tumour suppressor gene or the amplification of oncogenes.

1.4 Glioma biology

The aetiology of gliomas to date has been debated extensively. Although there is much that is unknown, a picture is emerging about the molecular events that lead to tumour initiation and progression. The WHO classification of the CNS tumours mentions studies undertaken in 1992 and 1997 of tumours arising from the malignant transformation of bipotential precursors cells or neural stem cells, respectively. The presence of Brain Tumour Stem Cells is widely accepted although debate still exists with regards to the efficiency of the enrichment markers used to demonstrate the entire stem cell population. In fact the previously thought most important stem cell marker CD133 may not be a stem cell marker but rather an epi-marker of proliferation [11]. Son and coworkers in 2009 have reported another enrichment stem cell marker, which seems to be more efficient. This marker is the stage-specific embryonic antigen 1 SSEA-1/LeX [12].

Currently the debate is about the origin of the stem cells and the mechanism which gives rise to a malignant tumour. There have been three theories postulated: the first holds that mature glial cells have acquired mutations that render their behaviour as "stem-cell like"; the second theory is that committed neural progenitors that have gone through a limited number of cell divisions and otherwise would terminally differentiate, acquire mutations that render their behaviour "stem-cell like". The third theory proposes that adult neural stem cells normally regulated, acquire mutations that render them able to create a tumour [13]. The last theory has been the basis of a study by Wang and his coworkers in 2009 [14] on a murine model featuring a mutant form of *p53* in a small population of neural stem cells located in the sub ventricular zone. These gave rise to mutated and fast dividing cells, suggesting that glioblastomas may originate by neural stem cells.

Glioblastomas are heterogeneous tumours at the cellular level. These cells have different proliferative potentials and their behaviour depends on their microenvironment as well as cell-to-cell interactions and genetic characteristics. The important genetic factors include the group of tumour suppressor genes and oncogenes that play an important role in the development of gliomas and their progression to higher grades. The capacity to proliferate depends on the cell cycle time, growth fraction, tumour doubling time and cell death. Cell death happens either by apoptosis (programmed cell death) or by necrosis, due to outgrowth of the tumour volume in relation to its vascular supply and by default to its nutrient and oxygen supply.

1.4.1 Tumour suppressor genes in Gliomas

Tumour suppressor genes are genes that regulate normal cell cycle progression and other aspects of cellular metabolism. During gene transcription they stop the cell cycle either by inducing apoptosis or by initiating DNA repair. The loss or inactivation of those genes leads to tumourigenesis. Although at a cellular level both alleles of the gene have to be lost or inactivated before this

contributes to tumourigenesis, at an organism level the number of cells at risk and the frequency of acquired loss or inactivation of the gene are high enough to greatly increase the chance of a tumour developing [15]. Examples of tumour suppressor genes are *Rb* (transcriptional co-repression), *TP53* (transcription factor), *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) and *ARF* (MDM2 antagonist) [16].

Retinoblastoma Rb

Knudson first suggested the existence of tumour suppressor genes after analysis of the incidence of familial retinoblastomas. This is a paediatric tumour syndrome often associated with pineoblastoma, meningioma, glioma or osteosarcoma. He reported that in order to develop retinoblastomas there is the need for two somatic mutations – the two hit hypothesis. In the familial form, however, those individuals who have inherited one mutant allele are predisposed to developing these tumours [17]. The *RB* locus is located on the long arm of chromosome 13 (13q14) [18]. Cyclin Dependent Kinase CDK inhibitors of the INK4 family, with D-type cyclins control this pathway. The protein pRb acts as a negative regulator of cell proliferation at the G1/S checkpoint. The downstream targets of the Rb pathway are the E2F transcription factors. Most human malignant tumours contain alterations of the genes in the Rb pathway either through inactivating mutations of the Rb1 gene or activating mutations and amplification of the D-type cyclins and CDK4 or CDK6. 13% of glioblastomas have been reported as having deletions of the Rb1 gene [19]. It has also been found that tumours displaying abnormalities of the Rb pathway commonly have *TP53* abnormalities [20]. These two pathways are intrinsically linked to each other and to cell proliferation.

TP53

The *TP53* tumour suppressor gene is located on the short arm of chromosome 17. Its loss or inactivation contributes to the development of colon, breast, lung carcinomas as well as gliomas [21]. It encodes for a 53 kDa nuclear phosphoprotein, which contributes in regulating cell division. The p53 protein is produced in response to cellular stress, hypoxia, irradiation, drug-induced genotoxic damage and oncogenic activation [22]. This protein binds tumour promoting viral proteins and suppresses transformation of cells by *myc* and *ras* oncogenes. It binds and activates specific genes that promote apoptosis or programmed cell death. Up to date there have been three apoptotic promoting pathways that have been linked to its DNA binding domain: 1) binding to Bcl2 family proteins at mitochondrial level; 2) binding to genes that promote apoptosis (microDNA, miR-34 gene family); 3) binding to Drosha complex to promote formation of microRNAs that suppress proliferation (miR-16-1 and miR-143) [23].

Point mutations of this gene are the most frequent genetic changes found in human malignancies. These almost always occur in the area occupied by exons 5 through exon 8. These point mutations occur in highly conserved sequences of the gene, suggesting that these sequences are vital for the function of the p53 protein. Point mutations involving exons 5, 7 and 8 that inactivate *TP53* have been found in astrocytomas [24]. The incidence of *TP53* mutations reported in secondary glioblastomas reaches 65% and is significantly more frequently seen than primary glioblastomas (10%) [25].

TP53, p14ARF and MDM2

P14 protein binds to the TP53/MDM2 complex inhibiting the MDM2 mediated p53 degradation. This indicates that p14 is an upstream regulator of the p53 protein. [26] P14 also acts as an inhibitor of MDM2. The result of the p53/MDM2/p14 interactions is a reduction of p53 concentration, which finally leads to tumour growth.

PTEN

PTEN was identified in 1997 by Li and coworkers [27], and has been located on 10q23 [28]. It encodes for a protein called phosphatidylinositol-3,4,5-triphosphate 3-phosphatase and was the first lipid phosphatase to be identified as a tumour suppressor [29]. It negatively regulates the Akt signalling pathway that is important for several cellular functions (nutrient metabolism, cell growth, apoptosis and survival) [30]. This mutation has been identified in 45% of glioblastomas [31].

1.4.2 Oncogenes in gliomas

Oncogenes are genes that act in tension with tumour suppressor genes to regulate many aspects of cell metabolism. In general they promote cell cycle progression and nuclear division as well as stimulate other metabolic pathways leading to cell growth. When overexpressed they lead to tumour development. They are involved in the control of the cell growth and can be classified into five groups as growth factors, growth factor receptors, signalling intermediates, transcription factors and modifiers of apoptotic pathways.

Growth factors and Growth factor receptors

Growth factors are molecules that occur naturally and stimulate growth, proliferation and cell differentiation. They act as signalling molecules between cells. They usually bind to cell surface receptors of their target cells and activate a signalling pathway, which leads to the specific effect of the growth factor.

The growth factors commonly found in brain tumours are members of the tyrosine kinase pathway. They bind to their receptors, initiating a signalling cascade that leads to proliferation. The growth factors and their receptors may be produced by the same cell resulting in autostimulation (autocrine production), or may be produced by adjacent cells (paracrine production) even of a different cell type (juxtacrine production) [32]. Finally, growth factors can also stimulate the production of other growth factors. The growth factors that have been reported to act as oncogenes in gliomas are the Epidermal growth factor (EGF), the Vascular Endothelial growth factor (VEGF), the Transforming growth factor-α (TGF-α), the Fibroblast growth factor (FGF), the basic Fibroblast growth factor (bFGF) and the Platelet Derived growth factor-α and –β (PDGFR-α and PDGFR-β) [33].

Growth factor receptors are cell surface receptors with three domains: an external domain which binds the growth factor, a second transmembrane domain which enhances enzymatic activity and the cytoplasmic domain which is part of the tyrosine kinase pathway and activates secondary signalling molecules by phosphorylating their tyrosine residues. The growth factor receptors themselves can be activated by a sequence mutation causing constitutive activation or over expression causing loss of growth control. Currently the most promising targets for therapeutic interventions in CNS tumours are the tyrosine kinase PDGFR and the VEGFR [34].

Epidermal Growth Factor (EGF) and EGFR

EGFR is a member of the Tyrosine kinase family and was the first of the human EGF receptor to be discovered. The *erb-B* gene encodes for EGFR. Wong and coworkers have demonstrated that over 80% of astrocytic tumours and all established astrocytic cell lines have higher levels of EGFR than normal brain [35]. The cascade activated by the ligand binding the receptor can be produced by four different mechanisms: i) increased expression of ligands, ii) *EGFR* gene amplification, iii) over expression of *EGFR* wild-type or mutant family members or iv) by *EGFR* independent mechanisms. The receptor has also been reported to undergo deletion mutations [36]. One deletion mutation removes sequences for down-regulation leading to the constitutive activation of the receptor. A second frequently observed deletion mutation results in the formation of the receptor with increased auto-phosphorylating activity. The third most common deletion mutation shortens the extracellular domain of the receptor. Mutant proteins which result from these deletions may be abnormally active and stimulate cell proliferation [37]. Gene amplification of *EGFR* has been found to be higher in primary (40%) rather than secondary (8%) glioblastoma. The presence of EGFRvIII in primary malignant gliomas is an independent predictor of poor survival [37, 38].

Platelet- derived Growth Factor (PDGF) and PDGF Receptor (PDGFR)

PDGF and PDGFR are also members of the Tyrosine kinase family. The processes regulated by them include cell proliferation, differentiation and migration [39]. Uhrbom and coworkers have shown that PDGF is involved in early tumourigenesis [40]. Other studies have shown that 75% of malignant gliomas have an over expression of PDGF [41].

Vascular Endothelial Growth Factor (VEGF)

VEGF is part of the Tyrosine Kinase family. It has specific action on endothelial cells, mediating vascular permeability, inducing angiogenesis and vasculogenesis. It promotes endothelial cell growth, cell migration and inhibits apoptosis. *VEGF* and *VEGFR* are over expressed in malignant gliomas and their levels have been positively correlated with increasing malignancy, vascularity and worsening prognosis [42, 43].

Transforming Growth Factor (TGF)

The transforming growth factor family is made of several proteins, which share the same morphology. They are all homodimeric polypeptides affecting cell growth, embryogenesis, cell differentiation, cell migration and adhesion, morphogenesis, immune cell activation, wound healing and cell survival. There are three isoforms of TGF-β that are expressed in gliomas [44]. TGF-β modulates bFGF, EGF and PDGF. Those together have a synergistic effect on cell proliferation, cell migration and invasion [45]. Glioma patients with high TGF-β have been shown to have poor prognosis [46].

Fibroblast Growth Factor (FGF)

This growth factor family has several members many of which have been found in the brain. Some of them induce angiogenesis and others are modulating their effects via the tyrosine kinase activity. bFGF has been found to be a key molecule in promoting astrocytic tumours and is present in virtually all CNS tumours including meningiomas, nerve sheath tumours, gliomas, and ependymomas [47].

Intermediate signalling pathways

These pathways relay information from cell surface receptors to the nucleus. They all have effects at transcriptional level. These are three main types: tyrosine kinases, G-proteins and IP3/DAG. The *ras* and the *src* oncogenes are also signalling molecules associated with development of brain tumours.

Ras is part of the protein family of small GTPases. Small GTPases are a family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP). When activated, it switches on other proteins, which turn on genes that regulate cell growth, differentiation and survival. Overactive *ras* signalling is an important mechanism in cancer development. Ras is a monomeric G protein, which gets activated by binding to GTP –the "on" status- and inactivated by binding to GDP – the "off " status. A mutated *ras* with a change in 3D conformation has reduced GDPase activity that renders *ras* constitutively active, sending continuously signals to the nucleus. It is involved in many overlapping signalling pathways having several effects. Mutations in *ras* have been found in several tumours including gliomas [48].

The protein src is a tyrosine kinase, which is located at the inner surface of the cellular membrane. When activated by a receptor, it initiates the tyrosine kinase cascade that has effects on cell growth, adhesion and cell motility. When it is not coupled it promotes tumour growth and cell invasion. The *src* oncogene has been found both in gliomas and in medulloblastomas [49].

Transcription factors in gliomas

Fos and c-myc are the two better-known transcription factors that promote tumour formation. They interact directly with the DNA altering the expression of several genes. Steroids and other signalling mediators activate these factors. They may form homodimers or heterodimers that bind to DNA and promote transcription.

Myc has been found to activate several factors that promote abnormal cell growth, proliferation, differentiation as well as metabolism [50]. Fujimoto and coworkers have reported that the level of *myc* expression positively correlates with the aggressiveness of gliomas and medulloblastomas [51].

Fos after forming a heterodimers with Jun regulates cell growth and proliferation. The Fos-Jun heterodimers binds to other factors to form complexes that promote transcription of genes for cell proliferation, differentiation, transformation and death [52]. 80% of malignant gliomas have been found to express *Fos* [53].

Modifiers of apoptotic pathways in gliomas

These are protein transcription factors that modify the cell's probability of entering the apoptotic pathway. *Bax* is a tumour suppressor gene and as such when lost the cell escapes from apoptosis. *Bcl-2* when over expressed acts as an oncogene, leading to prevention of cell apoptosis. Both these molecules affect apoptosis at a transcriptional level [54].

1.5 The Cell Cycle

There are two periods to the cell cycle: the first is called the interphase during which the cell grows, accumulates nutrients needed for mitosis and duplicates its DNA before proceeding into the second period consisting of the mitosis during which the cell splits itself into two "daughter cells". The interphase is divided into three phases G1, S, G2 followed by M.

The G1 phase, also called the growth phase, follows the mitosis. The cell produces several enzymes and prepares itself for the DNA synthesis needed in the next phase. The S phase is the time of DNA replication. G2 follows the S phase, and the cell can stay in that phase till it enters mitosis. The main products

of synthesis during this phase are the microtubules, which are vital for the completion of the mitosis.

The following phase is the Mitosis when the cell, which has double the amount of DNA, divides its nucleus into two identical nuclei. This is followed by the division of the cytoplasm, the organelles and the cell membrane resulting in the formation of two daughter cells that are identical between them and to the cell of origin.

The cell then will enter either into the G1 phase to synthesize all the enzymes needed for DNA replication but if there are no signals to commit the cell to divide, it will exit temporarily the G1 phase and will enter the G0 phase becoming quiescent.

Progression from one phase to another is very tightly regulated. This is crucial for cell survival, detection of genetic damage as well as prevention of uncontrolled division. The phases of the cycle are sequential, meaning that each phase has to be completed before progression to the following one and that the cycle is not reversible.

There are two groups of molecules that regulate the cell cycle, the cyclin dependent kinases (CDKs) and the cyclins. These form heterodimers that when bound together are active. Each of the two subunits of the heterodimers is inactive in the unbound state. When the complex forms, the CDK phosphorylates its target proteins a process, which will modify their activity status and will signal the progression to the next phase. Cyclins are synthesised and degraded in each cycle regulating the formation of the Cyclin/CDK complexes.

The first cyclin to be formed during the cell cycle is cyclin D. This binds to the CDK4, forming the active complex, which phosphorylates the pRb susceptibility protein. CDK4, when dissociated from the E2F/DP1/Rb complex renders the

E2F genes active. This promotes the transcription of several genes and the formation of proteins such as cyclin E, cyclin A, and DNA polymerase etc.

The cyclin E produced during this phase binds to the CDK2, forming the active complex which signals the progression of the cell cycle to the S phase. Another cyclin, cyclin b, when bound to the CDK1, initiates the transition of the cell cycle from G2 to M phase. When this complex is deactivated, the cell exits mitosis [55]. The cip/kip gene family, which includes p21, p27 and p57 genes, and the INK4a/ARF gene family that includes p16INK4a, are part of the tumour suppressor genes that are vital for the prevention of the progression of the cell cycle.

The cip/kip family binds and inactivates the cyclin/CDK complex arresting the cell cycle in G1 phase. Different molecules activate the various genes: p21 is activated by TP53, which is activated by DNA damage. P27 is activated by TGFb, which inhibits growth.

The INK4a/ARF family binds to CDK4 and also arrest the cell cycle into G1 phase. There are several checkpoints during the cell cycle during which damaged DNA is repaired and all necessary processes of the specific cell cycle have been completed prior to progression to the next phase of the cycle. The two main checkpoints are the G1/S checkpoint also known as restriction checkpoint and the G2/M checkpoint. The gene, which plays a major role in activating both the checkpoint mechanisms, is TP53 (Figure 1-1) and (Figure 1-2).

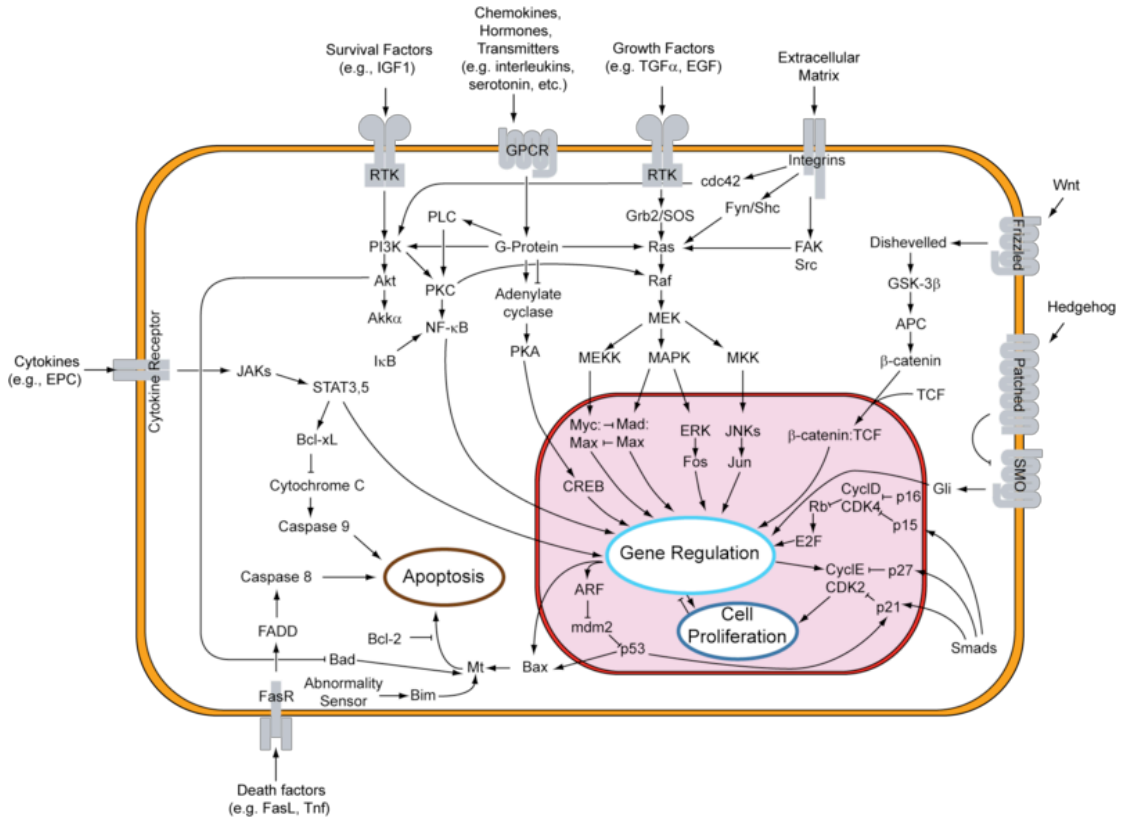

Figure 1-1 Apoptosis signal transducing pathways. Adapted from [56]

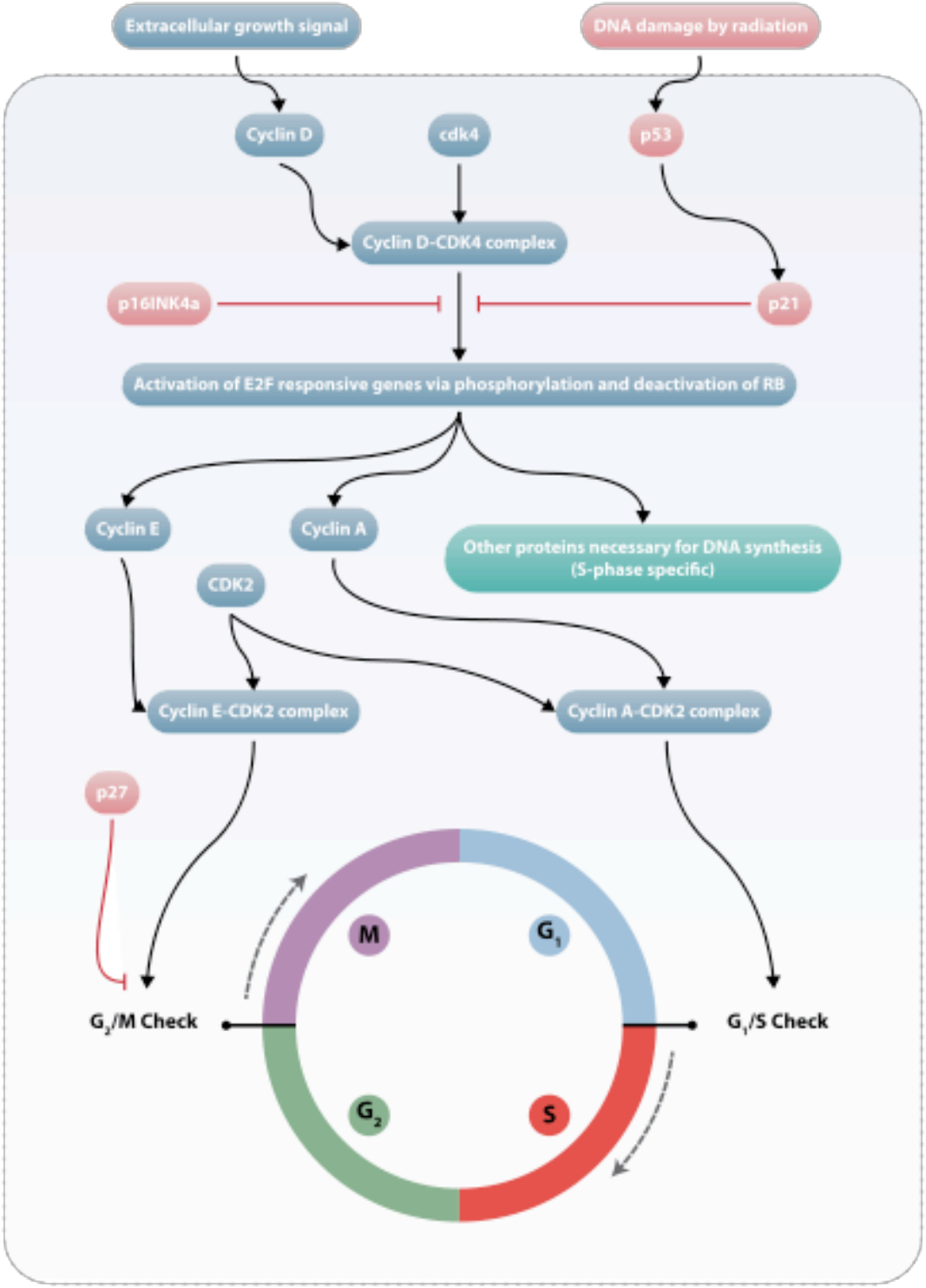

Figure 1-2 Regulation of the cell cycle. Adapted from [57]

1.6 Glioma Diagnosis

Early clinical diagnosis is a key prognostic factor for every cancer. Early diagnosis is most likely to be made on a patient who will have a better performance status, than someone diagnosed later during the disease process. The performance status of the patient, which is classified according to the Karnofsky score, is a major predictor of suitability for treatment appropriate for each patient. It is intuitive that a patient with a better Karnofsky score will be able to tolerate more aggressive multimodality treatment than one who is diagnosed later in the disease with a worse performance status. This is a major challenge for gliomas. The diagnosis is based on a careful history and examination of a patient who usually presents with slowly progressive symptoms with the exception of epilepsy and haemorrhagic tumours. The most common symptoms of a patient with a glioma are headache, symptoms of raised intracranial pressure such as nausea and vomiting, symptoms caused by the location of the tumour such as memory decline, sensory and or motor deficits, visual/auditory changes, and non specific symptoms such as depression and loss of appetite. New onset of seizures or a focal deficit is highly suggestive of the presence of an underlying lesion.

The next step in the diagnosis of patient with glioma is a careful physical examination of the patient. This will aid in trying to locate the lesion, as well as determining the performance status of the patient.

Imaging of the neuroaxis follows in the diagnosis. The initial screening is made with a computerised tomography (CT) scan, which is a rapid and relatively inexpensive imaging method. The CT highlights the presence of calcification, mass effect, oedema, haemorrhage and hydrocephalus. Although CT is very good in visualising supratentorial tumours, it is limited in the ability to highlight the presence of tumours in the cerebellum and brainstem.

Magnetic Resonance (MR) imaging is superior to CT in detecting, locating, and assessing tumour extent. It is though limited in its capacity in assessing tumour invasion to adjacent normal brain [58, 59]. Both imaging techniques can be combined with administration of contrast, which enhances the tumour mass as it "leaks" out of the disrupted blood-brain barrier.

More advanced MR techniques that combine anatomical information with cellular metabolism, such as MR spectroscopy (MRS), diffusion and perfusion MR, functional MR (fMRI), diffusion tensor imaging (DTI), are able to demonstrate the extent and the nature of gliomas as well as the relation of the lesion with eloquent areas of the brain. MRS detects freely mobile metabolites such as lactate, glutamine, creatinine, choline, lipid, and generats metabolic spectra. It is not widely used in clinical practice but it is helpful in detecting the most malignant part of the tumour that could aid surgical planning and to grade the glioma [60]. MR perfusion and MR diffusion acquire physiological data, while water suppressed proton spectroscopy MR acquires metabolic data, both providing measurements of biological properties of the tumour as well as changes in tumour vascularity, cellularity, proliferation and also progression to a higher grade [61].

Another imaging technique, which has proven useful in detecting tumour residue post treatment, is Positron Emission Tomography (PET). This imaging technique used 18-fluorodeoxyglucose to "light up" metabolically active cells [62]. PET is also very helpful in differentiating between recurrent tumour and radiation necrosis, which is vital for determining the correct management plan for the patient [63].

Dynamic susceptibility-weighted contrast-enhanced perfusion MR (DSC MRI) provides information about blood volume and blood flow of the tumour and complements conventional MR in evaluating angiogenesis and microvasculature. Dynamic contrast enhanced MR, detects the status of vascular and endothelial permeability. It has been reported that quantitative measurement of microvascular permeability correlates with the glioma grade

[64, 65]. Although not currently used widely in clinical practice, it potentially can help the surgeon to biopsy the most malignant part of the tumour; it can monitor the efficacy of chemotherapy especially when using antiangiogenesis drugs and also differentiate between radiation necrosis and recurrent tumour [59].

1.7 Prognosis of gliomas

Prognosis varies according to the glioma grade. Pilocytic astrocytomas (WHO grade I) have the potential for cure following total surgical resection.

Age, preoperative functional status and extent of surgical resection are the most important prognostic factors for Grade II astrocytomas. Survival rates are improved in patients with lesions that can be totally resected. Philippon and coworkers have reported that the 5-year survival rate decreases dramatically from 80% for patients who underwent gross total resection to 50% for those who had partial resection and to 45% for those biopsied only [66].

Prognosis for malignant gliomas (anaplastic astrocytomas WHO grade III and glioblastoma multiforme WHO grade IV) remains dismal, despite pharmacological advances in chemotherapeutic agents and technical improvements in both neurosurgery and radiation delivery. In fact, the goal of treatment is to improve quality of life rather than cure. Again, in this group of gliomas, age, preoperative functional status and histological grade are the main prognostic factors. The gold standard of therapy for the anaplastic astrocytomas is macroscopic total resection, followed by radiation therapy. Chemotherapy treatment remains controversial [67, 68]. Median survival is 2-3 years, with 18% 5-year survival [69].

Glioblastoma multiforme has an average survival of less than a year even following optimal aggressive multimodality therapy. 5-year survival is less than 5%. The gold standard of treatment for glioblastoma multiforme is radical

resection, followed by radiotherapy and chemotherapy. The younger the patient and the better the performance status, the longer the time to progression and the longer the overall survival [70-72].

1.8 Treatment of gliomas

The treatment of gliomas is multimodal and includes surgery, radiotherapy and chemotherapy. The extent of surgery, mode of radiotherapy and type of chemotherapy is dependent on the histological diagnosis of the glioma, as well as the clinical condition of the patient. Currently a combination of all these modalities is given to suitable patients. Unfortunately these tumours are incurable and the aim of therapy is to prolong high quality survival.

1.8.1 Surgery

Surgical options are biopsy, subtotal or gross total resection. The purpose of the surgical intervention is to provide tissue for definitive diagnosis, alleviate symptoms of raised intracranial pressure and provide maximal cytoreduction of malignant cells. The type of surgical intervention will depend on the location and size of the tumour, presence of mass effect or increased intracranial pressure, the patient's condition and the risk of surgery. Complete resection of the tumour is impossible due to its infiltrative nature. Studies have shown that the survival increases with the increase of the extent of surgical resection [73, 74]. All surgical modalities provide diagnostic clarification of the tumour in 98% of patients. Gross total resection may also provide relief of symptoms and signs of increased intracranial pressure as well as improve neurology and functional status of the patient. It may also improve tolerability during radiotherapy treatment due to reduced steroid requirements.

Surgical planning is one of the most important steps of the surgical treatment. It should be tailored to individual patient need, maximising the extent of resection

whilst minimising the morbidity of the procedure. Although there has been development in the surgical techniques, which have helped in achieving minimal risks during surgery, the survival of patients has not improved [75].

For non-eloquent areas of the brain affected by a glioma, the traditional craniotomy under general anaesthesia is widely used. This has been enhanced with the use of image guidance. However, for patients whose eloquent areas of the brain are affected by gliomas, pre operative functional MR, fusion of functional and anatomical imaging and neuronavigation, are vital adjuncts to the surgeon to prevent new neurological deficits [76]. Furthermore, the development of better anaesthetic drugs as well as techniques and intraoperative functional monitoring has enabled the safe performance of awake craniotomies with even better results in terms of extent of excision with minimal neurological deficits [77]. One adjunct, which allows maximal resection of tumour around the motor cortex is monitoring of the corticospinal motor evoked potential (D-wave). When the amplitude of the D-wave decreases by less than 30%, this is suggestive of postoperative preservation of motor function [78]. Use of intra-operative MR has been demonstrated to maximise tumour resection [79]. Also the possibility to fuse functional MRI with neuronavigation helps prevent development of new motor deficit after surgery. It has been reported that a safe lesion–to-activation distance is 10mm for complete resection [80].

1.8.2 Radiotherapy

Radiotherapy is the primary adjuvant treatment after surgical resection of gliomas. It uses x-rays/ γ -rays to damage the DNA of dividing cells and cause cell apoptosis. Standard treatment dose for glioma is whole brain radiation with 60 Gy using conventional external beam radiation, in fractionated doses. This treatment usually starts a couple of weeks after surgery, allowing the wound to heal. Although this treatment has been reported to increase survival of patients with grade III and grade IV gliomas regardless of the extent of surgical

resection, the mean survival time is still disappointing, ranging from 16-70 weeks [81, 82].
Radiation treatment for low-grade gliomas, on the contrary, is controversial mainly because of the long-term side effects of radiation to the normal brain and the young age of patients [83, 84].

To maximise the effects of radiation therapy to the malignant tissue, minimising its effects to surrounding normal brain, which has minimal tolerability to x-rays, Woo and coworkers, have reported on novel ways of delivering radiation therapy to the tumour bed, sensitizing the tumour cells to the effects of radiation either chemically or thermally, or using alternative radiating particles such as neutrons or heavy ions [85].

The use of stereotaxy has revolutionised delivery of radiation therapy. Stereotactic radiotherapy is delivered by external beam. External beam can be fractionated and deliver an even dose of radiation to a specific area or can be delivered as one-session using intensity modulated radiation therapy which shapes the beam to approximate the tumour as closely as possible, avoiding damage to adjacent structures. This modality of radiotherapy is widely known with the various brand names as Gamma Knife, Novalis Tx, CyberKnife radiosurgery [86].

Brachytherapy involves the placement of radioactive implants to the tumour that emit radiation for a prolonged period of time. This type of radiotherapy is called interstitial brachytherapy when the implants are placed in the tumour bed, or intracavitary brachytherapy, when the tumour is cystic or the implants are placed to existing anatomical cavities. Using permanent, low-activity implants has been reported to improve outcome, without the risk of side effects and that of radiation necrosis [87].

An emerging novel technique of delivering radiotherapy is Radio-immunotherapy. This technique uses monoclonal antibodies labelled with radioactive ions, to selectively irradiate tumour rather than normal cells [88].

The side effects of radiotherapy treatment can be grouped as acute, early-delayed and late-delayed side effects. The early side effects occur during treatment and are readily responsive to steroids. These include reversible neurological deficits due to tissue damage. Early-delayed side effects include nausea, vomiting, dysphagia, cerebral and cerebellar dysfunction, which are presumably due to transient demyelination. These occur weeks to months after treatment. The late-delayed side effects are non-reversible cerebral or cerebellar dysfunction and are the result of radiation necrosis. These occur six months to years after the end of treatment [89].

1.8.3 Chemotherapy

Chemotherapy uses cytotoxic drugs to kill or render tumour cells radiosensitive. These are delivered either systemically using the oral, intravenous and intraarterial route, or locally using an intrathecal or intracavitary delivery. The blood-brain barrier (BBB) protects the brain from most chemotherapeutic regimens, limiting the drug choice and method of delivery of those drugs for the treatment of gliomas.

The use of post-operative chemotherapy has been correlated with an increase in the survival of patients with malignant gliomas [90]. The drugs are classified as cell cycle specific and non cell cycle specific.

Cell cycle specific drugs include microtubule modulators, which derive from plant alkaloids. Microtubules are dynamic complexes made from the protein tubulin. These complexes promptly polymerize and depolymerize in cells while undergoing two dynamic processes called dynamic instability and treadmilling. Microtubules are crucial during the mitosis of the cell cycle as they attach to each chromosome and guide it towards the new forming daughter cell, making sure that the correct numbers of chromosomes end up in the correct cell.

The main microtubule modulator drugs are vinca alkaloids and taxane alkaloids. Vincristine, vinblastine and vinorelbine are examples of vinka alkaloids. Those inhibit the polymerization of the microtubules that impedes mitosis. Vinorelbine is a second-generation semi synthetic vinka alkaloid, which has different binding site from that of the natural products Vincristine and Vinblastine and shows increased antitumour activity with reduced neurotoxicity and reduced affinity for p–glycoprotein [91].

The group of taxane alkaloids such as paclitaxel and docetaxel stimulate the polymerization of the microtubules stabilizing them, hence prohibiting the crucial dynamic instability of the microtubules during the process of mitosis. Both groups lead to cell death via apoptosis [92].

Paclitaxel has been very effective in treating various cancers and is used currently in breast, lung and ovarian carcinomas. It is a highly hydrophobic compound, which binds to the b-tubulin subunit of microtubules at the M-loop stabilising lateral contact between protofilaments. As it is hydrophobic, it requires solubilisation in an alcohol-based molecule. This is thought to be the base of the adverse reaction that it causes to patients. It is unfortunately also a good substrate for the p-glycoprotein. P- glycoprotein is a plasma membrane protein, which acts a drug efflux pump. Paclitaxel is bound and pumped out of the cell, abolishing its effects. There have been reports of *p53* transcription inactivity increases sensitivity to microtubule modulating drugs. This is mediated by the regulation of microtubule-associated protein 4 (MAP4), which when abundant increases microtubule polymerization and binding to paclitaxel. Expression of MAP4 is down regulated by *p53* transcription [93].

Non-cycle specific drugs include Topoisomerase inhibitors and alkylating agents. Topoisomerase inhibitors include etoposide, tenisopide, irinotecan and topotecan. Etoposide causes single and double strand DNA breaks that are dose dependent, at concentrations used *in vivo* [94]. Topoisomerase II and I are enzymes expressed only in dividing cells during specific mitotic phases.

Alkylating agents are the second group of non-cycle specific chemotherapeutic agents used for the treatment of malignant gliomas. These agents act by attaching an alkyl group (CnH2n+1) to DNA. This causes crosslinking of the guanine nucleobases in the double-helix strands of the DNA, making them unable to uncoil, a process, which is necessary for replication. The end result is the death of the cell.

There are three groups of alkylating agents: the nitrogen mustards; the nitrosoureas and the alkyl sulfonates. The nitrogen mustards include agents like cyclophosphamide, chlorambucil and melphalan. These agents were the first alkylating agents to be used in medicine. Prior to their use in chemotherapy, they were used as chemical weapons in World War I.

Nitrosoureas are commonly used for the treatment of malignant gliomas. Carmustine (BCNU) and Lomustine (CCNU) are examples of these agents. These are dialkylating agents as they act in two different 3-7-N Guanine nucleobases causing crosslinking of the DNA strand, if the residues are located in two different strands of DNA. If the residues are on the same strand, the effect of the drug is called limpet attachment. This does not prevent the separation of the DNA helix, but renders the DNA inaccessible to the enzymes required for replication. As a result, the cell enters apoptosis and dies.

Temozolomide is another alkylating agent, which has revolutionised the treatment of malignant gliomas over the last ten years as it increased overall survival by 2-3 months. It is an imidazotetrazine derivative, which attaches a methyl group to the guanine nucleobase of DNA, preventing its replication and causing cell death [95]. It mediates its action by it active metabolite, which forms spontaneously after administration. It is a well-tolerated agent, which is administered via the oral route. Using adjuvant Temozolomide for six months after radiotherapy in the treatment of glioblastomas, known as the Stupp protocol, has shown an increase in survival over radiotherapy alone [96].

The DNA mismatch repair enzyme O6-methylguanine-methyl-transferase (MGMT), removes alkyl adducts from the O6 position of guanine and the O4 position of thymine, repairing the lesions caused by Temozolomide [97]. It has been reported that methylation of the MGMT gene promoter region leads to inactive enzyme production which increases survival of patients treated with Temozolomide after radiotherapy versus patients treated with radiotherapy alone [98, 99].

Platinum based chemotherapeutic agents are described as "alkylating-like" agents as act in a similar manner. They from adducts in the N7 position of the adenine and guanine nucleobases. This causes permanent DNA damage, blocking the action of the DNA polymerase, preventing cell replication and, leading to cell death. Another mode of action of cisplatin in human glial cell lines, which leads to apoptosis, is mediated by the activation of the ERK signalling pathway, which activates numerous upstream pathways and the caspase-3 pathway [100].

1.9 Alternative Therapeutic Modalities

As survival has not improved significantly with improvement in chemotherapeutic agents and radiation therapy, there has been an interest in developing other therapeutic modalities. These modalities include biological response modifiers, gene therapy, Oncolytic viruses, immunotherapy and inhibitors of angiogenesis. These therapies exploit the molecular differences between normal glial cells and glioma cells, but have yet to be proven effective.

1.9.1 Biological response modifiers

These agents can modify the phenotype of tumour cells. The most widely studied are the interferons (IFN), interleukins (IL) and tumour necrosis factor-α (TNF-α).

Interferons are cytokines that modulate immune response and tumour cytotoxicity. There have been reports of IFN-α and IFN-β inhibiting tumour growth in rodent glioma models. Yung and coworkers have reported that IFN-β is more active than IFN-α [101].

Interleukins are molecules that activate lymphocytes and leukocytes. Benveniste and coworkers have reported the anti-glioma *in vitro* activity of IL-2 [102].

1.9.2 Gene Therapy

The lack of systemic toxicity and the ease of application during surgery make gene therapy an attractive treatment modality for brain tumours. Genes can be introduced to the DNA of the target cell either directly or indirectly (Figure 1-3). A direct introduction of genetic material without any vector (cellular or viral) can be achieved via systemic delivery, aerosol or microcellular injection. Indirect gene delivery can be performed via transplantation of genetically engineered cells, or via inoculation of recombinant defective virus into the target DNA [103].

There have been studies where an adenovirus is used to deliver the herpes simplex virus thymidine kinase (HSV-tk) gene to the dividing glioma cells. The kinase that is produced in the infected cells blocks DNA replication by phosphorylating nucleoside analogs to form nucleotide-like precursors [103].

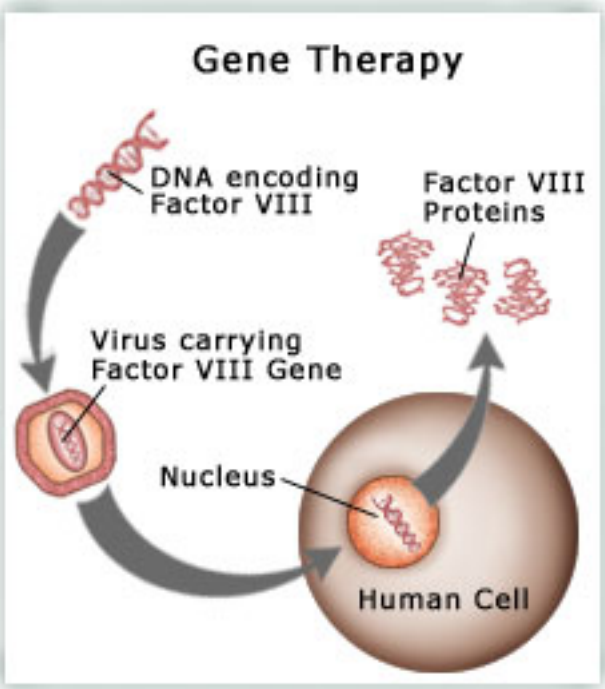

Figure 1-3 Mechanism of gene therapy. Adapted from [103]

1.9.3 Oncolytic viruses

Oncolytic viruses replicate selectively in neoplastic tissues. This has a result the lysis of the neoplastic cell (Figure 1-4). There have been both *in vitro* and *in vivo* studies that have demonstrated that certain strains of reovirus can take advantage of the Ras-signaling pathway of human gliomas to replicate [104]. Ikeda and coworkers have demonstrated that human immune response impairs viral activity [105]. It remains to be seen if the oncolytic viral therapy will translate into an alternative therapeutic modality for malignant gliomas.

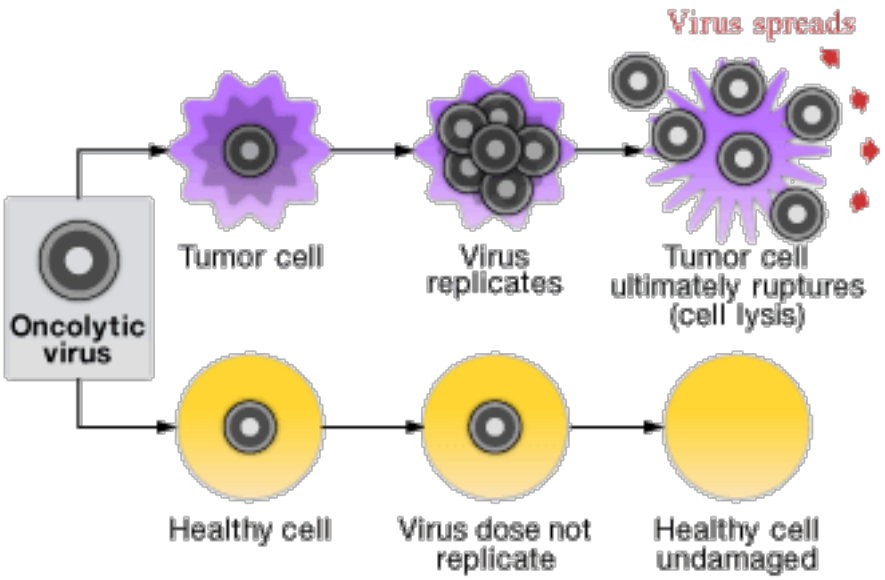

Figure 1-4 Mechanism of action of oncolytic virus. Adapted from [106]

1.9.4 Immunotherapy

Immunotherapy is an exciting therapeutic modality for the treatment of brain tumours. Its specificity and the memory of the immune system could potentially eliminate infiltrating tumour selectively without damaging the surrounding normal brain.

Passive, active and adoptive immunotherapy are currently under investigation as a means of therapy [107].

Passive immunotherapy employs the use of monoclonal antibodies in order to induce tumour cell death either directly or by delivering radiotherapy or toxins [108].

Active immunotherapy utilises vaccination with reproductive incompetent autologous or even allogeneic tumour cells in order to induce a specific anti-tumour response [109].

Adoptive immunotherapy induces response either by *ex vivo* stimulation of the patient's own immune effector lymphocytes or by injection of activated LAK+ IL-2 directly into the tumour via an Ommaya reservoir [110, 111].

1.9.5 Inhibitors of Angiogenesis

Neoangiogenesis is a cardinal feature for tumour survival and is required in order for the tumour to increase in size by more than 1-2 mm in diameter. This is possible through a continuous process of balancing regulators of angiogenesis towards promotion and absorption of new blood vessels.

The extracellular matrix over expresses and mobilises angiogenetic proteins as well as cells such as macrophages which themselves produce angiogenetic proteins. Vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF) and platelet-derived growth factor (PDGF) are the cardinal angiogenic proteins. Angiostatin, endostatin and thrombostatin are endogenous inhibitors of angiogenesis.

During angiogenesis, a series of events occurs such as activation of endothelial cells, proteolytic degradation of the extracellular matrix and the basement membrane, proliferation and migration of endothelial cells, endothelial tube formation, fusion and reassembly of the extracellular matrix. These processes involve many enzymes, including the matrix metalloproteinases, the serine proteases and the cathepsins, which promote release of stored growth factors, which in turn promote more angiogenesis.

The more studied enzymes in the antiangiogenesis treatment of brain tumours are the matrix metalloproteinase inhibitors (MMPIs). The MMP family is a group of twelve secreted as well as membrane-bound zinc endopeptidases. Other proteolytic enzymes activate them in order to digest the extracellular matrix.

MMPs are upregulated in both primary and secondary brain tumours and correlate with malignant progression as shown by Van Meter and coworkers [112]. However clinical trials have failed to demonstrate good therapeutic efficacy.

1.9.6 Biomarkers

There has been growing interest in identifying biomarkers for malignant gliomas. A biomarker is a measurable substance, which can used to indicate a biological state. Such state may be a normal biological process, a disease or even a pharmacological response to treatment. Examples of commonly used biomarkers include Hb_1Ac for diabetes mellitus, blood pressure monitoring in hypertension, and chromosomal abnormalities in genetic syndromes.

Molecular biomarkers are widely used for disease monitoring in several common cancers, such as prostate cancer, ovarian cancer and hepatocellular cancer. In recent years, several studies have identified potential candidate molecules as brain tumour biomarkers but so far very few of them are used in clinical practice [113]. Amongst the most promising ones for clinical use are the Cytokines and Angiogenesis factors [114] (see introductions of Chapter 4 and 5).

1.10 Aims of the following study

The aims of the present study are two fold. Firstly it seeks to evaluate the use of *in vitro* chemosensitivity assays in primary glioma cell cultures. Secondly it aims to identify novel brain tumour biomarkers from serum samples of patients affected by malignant brain tumours.

In vitro chemosensitivity testing is a well-established idea. However, it has failed to make an impact in routine clinical practice. This study aims to correlate the results of chemosensitivity testing with clinical data of treatment and survival in an attempt to "validate" the *in vitro* findings and assess the potential use of this in clinical practice.

Although several molecules have been proposed as potential brain tumour biomarkers, none so far has been found to be useful in clinical practice. This study investigates the feasibility of collecting blood samples from patients and controls in a clinical setting. It also tries to identify possible molecules that could serve as malignant brain tumour biomarkers. It acts as a pilot study for further projects in this field in our institution.

2 CHEMOSENSITIVITY TESTING IN PRIMARY GLIOMA CULTURES

2.1 Introduction

Chemosensitivity is defined as the susceptibility of a microbial pathogen to the action of a chemical agent. Medical oncology has adopted this concept in order to investigate the responsiveness of cancer cells to various chemotherapeutic agents [115]. Successful implementation of this notion could then lead to personalised chemotherapy treatment where tailored drug-dose combination is administered soon after the chemosensitivity testing. This could potentially maximise the chances of survival for the cancer patients.

Chemosensitivity studies are mainly performed on cell cultures. These can be either primary cell cultures or cell lines (aka immortalized cell lines). Primary cell cultures derive directly from the parent tissue and have the same karyotype and chromosome number as the cells of the tissue of origin. Most primary cell cultures have a limited lifespan, which is approximately less than 19 passages. On the other hand, cell lines have been derived from primary cell cultures that can proliferate indefinitely given appropriate fresh medium and space. The genetic make up of the cell lines differs from that of the primary cell cultures as well as their tissue of origin.

The first successfully cultured human malignant tumour cells were the HeLa cells, which were produced by a patient with cervical cancer [116]. There is an ongoing scientific debate with regards to how representative these cell lines are of the original tumour. It is inevitable that with every cell passage there is clonal selection of rapidly dividing cells. Therefore, subsequent cell populations will carry only a fraction of the genetic heterogeneity that was originally present in the primary cell culture. These genetic differences in cell lines present a limit as to how useful these cultures are in research and consequently in clinical studies. In the case of human gliomas, there are currently more than 30 known cell lines.

Given the above limitations of the cell lines there is a growing trend towards using primary glioma cell cultures. In addition, primary glioma cultures are more representative of the considerable genetic heterogeneity of tumours between patients and therefore more suitable for chemosensitivity studies. Currently primary glioma cultures are routinely produced in the laboratory albeit with varying degrees of success [117].

Primary culture is an established and successful method of drug testing in several disciplines; however, there are a few limitations when used to test chemosensitivity in glial tumours. This is mainly due to the fact that the laboratory conditions do not resemble the *in vivo* environment of the tumour bed [118, 119]. For example, tumour cells in flasks grow as a monolayer, which represents partly the natural form of cell growth. In addition, this monolayer of cells lacks the supportive network of other cells such as microglia and neurons. Furthermore, primary cultures growing in flasks are feeding on growth medium rather than on nutrients from the blood supply.

An important consideration when using primary cultures is the fact that the brain is an immunologically privileged organ. The cells, which provide this role, are not present in this type of *in vitro* environment. As such primary cultures are deprived from any immunological interaction.

Another limitation is the type of drugs that can be used for chemosensitivity testing. These drugs need to act directly on the primary tumour cells (i.e. cytostatic or cytotoxic). Drugs that act on a different target, such as on the blood supply and vasculature, would not be effective when tested *in vitro*. The same applies for drugs that require activation in a remote site such as the kidney or liver [120].

As mentioned previously, the cells in the culture have to be representative of the original tumour. However, as the most proliferative tumour cells will be forming the confluent monolayer, malignant quiescent cells will not be included

in the chemosensitivity testing. Performing the chemosensitivity testing directly onto the fresh tissue specimen could potentially alleviate such misrepresentation [121]. However, the specimen that arrives in the laboratory also contains other cells types as well as debris. These include cells such as erythrocytes, leucocytes, epithelial, endothelial and normal neuronal cells, as well as other tissue and bone dust from the craniotomy. Given this mixture of cells and material, it would be very hard to correlate the results of the chemosensitivity assay with the cell type responding to the drug tested.

As mentioned above, chemosensitivity testing could be a useful method in predicting *in vivo* response to cancer treatment. For example, ovarian, colon and breast cancer patients treated with drugs that have shown *in vitro* effectivity showed an improved overall survival [122]. Conversely, when leukaemia, colon and gastric cancer patients were treated with drugs that were resistant *in vitro*, their overall survival was poorer [123]. Moreover, Pieters and coworkers have shown that *in vitro* chemosensitivity testing is more important than any other laboratory or clinical variable in predicting what form of chemotherapy is to be used [124].

Kronblith and Szypko (1978) were amongst the first to perform chemosensitivity testing in malignant gliomas. More specifically, using the *in vitro* Giemsa staining, they tested 24 malignant glioma cultures with Carmustine (BCNU) and found that 18 out of 24 cultures were sensitive at 33μg/ml [125]. Other studies went one step further and correlated the *in vitro* chemosensitivity results of glioma cultures with the survival benefits of their donor patients. For example, Bogdahn treated 17 cultures with Carmustine using the ^{14}C leucine and ^{3}H uridine uptake method [126]. He reported that 7 out of 17 sensitive patients had a recurrence free survival (RFS) of 15 months. On the other hand, the remaining 10 patients who were resistant to Carmustine had a RFS of 6 months [126]. In another study, Rosenblum treated 10 malignant gliomas with BCNU. Using the colony-forming assay he reported that 4 out of 10 primary cultures were sensitive *in vitro*. Of those four patients, 3 patients responded with a prolonged RFS [127]. A few years later, Darling and coworkers tested 99

glioma cultures with Procarbazine, Lomustine (CCNU) and Vincristine using an amino acid precursors uptake assay [128]. Only thirty-three out of those 99 tumour patients had clinical data available. They reported that when patients, whose tumours were sensitive in-vitro to CCNU, were treated with the drug had a longer overall survival. Furthermore, no correlation was observed between survival time and the other two drugs used.

As seen above, a number of different assays have been used to measure chemosensitivity. A brief overview of the most commonly used assays is provided in the section below.

2.1.1 Micro Cytotoxicity Assay

This assay was one of the earliest to be used in chemosensitivity testing in malignant gliomas. It measures the ability of the malignant cells to remain attached to the culture petri dish after they have been exposed to the various drug concentrations tested. Briefly, the malignant cells are plated in a micro titre plate and are exposed to the various drug concentrations for a fixed period of time. Following this, the drug is washed out and incubation for a further fixed period of time with a drug free medium takes place. The plate is then stained with 4% Giemsa stain and the number of the attached cells to each well is counted. This measurement is used to generate a drug response curve [129]. The major drawback of this assay is that it does not distinguish between alive and dead cells and as such more often than not, sensitivity is underestimated. Furthermore, a wide variation between wells treated with the same drug concentration is observed as only a small number of cells are plated into each well.

2.1.2 Colony Forming Assay

Salmon and Hamburger first used this assay when attempting to measure the growth of a stem cell population [130]. A single cell suspension of the tumour

tissue sample was plated on a soft agar or as a monolayer on a culture flask. This was left to incubate for 2-4 weeks. During that period, some cells would have formed a colony of minimum 50 cells, having gone through at least 6 cell cycles. This would imply a substantial proliferative activity. The colonies were treated with the test drug concentrations and the number and size of the treated colonies was compared with the untreated ones. An arbitrary figure of 70% inhibition of colony formation was taken as the cut-off to mark sensitivity. The drawbacks of this assay are as follows: Firstly the technical difficulty in obtaining the single cell suspension [131]. Secondly, the possibility that some of the colonies are derived from pre-existing groups of cells. And thirdly, it may not measure quiescent cells, which may later enter the cell cycle. On the other hand, this is one of the few assays available able to measure *in vitro* radiation sensitivity.

2.1.3 Radiolabelled Assays

These assays measure the inhibition of incorporation of radiolabelled precursors during the synthesis of DNA, RNA and proteins. Such precursors can be thymidine, uridine and amino acids. The measurement of the amount of radioactivity incorporated into a substrate as a function of time and enzyme concentration allows enzyme activity to be quantified [132].

During these assays, cells are exposed to various drug concentrations and allowed to recover for a fixed period of time. They are then exposed to the radioactive precursor and the resulting uptake of radioactivity is measured.

Overall, these assays are inexpensive, sensitive, accurate, relatively easy to use and provide results within a short timeframe. However, there are a few drawbacks, which can potentially provide inaccurate chemosensitivity results. Firstly, inhibition of synthesis or replication can be a temporary effect [133]. Secondly, cells that receive a potential lethal drug concentration can still undergo several cell cycles prior to their death. And thirdly, these assays do not

account for quiescent cells as they only measure cells in the S or G1 phase of the cell cycle.

2.1.4 Multicellular Tumour Spheroid Assays

These assays use tumour spheroids grown in soft agar or serum free media. The spheroids can be picked up individually and analysed histologically after fixation and staining. These assays are useful as tumour models in studying cell-to-cell interactions and drug penetration [134].

2.1.5 Colorimetric assays

This group of assays have been widely used for chemosensitivity testing in malignant gliomas [135]. Amongst them, the most popular one is the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay [117, 136]. In this assay, the MTT is converted by mitochondrial enzymes to a coloured insoluble formazan product. This product is then solubilised in alcohol or a detergent and the absorption is measured. Recently this assay has been simplified by avoiding the second step of solubilisation when a one step reagent is used [137]. The main drawback of this assay is that the number of tumour cells limits its sensitivity.

2.1.6 Luminescent assays

Bioluminescence-based assays are widely used in several biological and medical disciplines and are based on the measurement of ATP in living cells. Bioluminescence is detected by the interaction of a substrate luciferin and ATP together with an enzyme called luciferase, which produces light. More specifically, luciferase catalyses the mono-oxygenation of the compound luciferin to oxyluciferin in an ATP dependent fashion. The oxyluciferin

produced, is in an excited state that decays emitting a photon to reach its ground state.

The assay measures the emitted photon quantitatively in a luminometer. The measured luminescence is directly proportional to the quantity of ATP and as such to the number of cells. ATP concentration relates to cell number and it degrades rapidly after cell death. Measuring ATP would therefore provide an estimate of the biomass [138].

Chemosensitivity assays that are using bioluminescence have been reported as being more sensitive than other methods. In addition, they correlate better with patient response to treatment in breast and ovarian cancers when compared to other assays [139-141]. Petty and coworkers have demonstrated that the bioluminescence-based ATP assay is able to down to 1500 cells per well where the MTT assay could not detect fewer than 25.000 cells per well [142]. Work performed previously in our laboratory has demonstrated that this assay can reliably assess chemosensitivity in gliomas even with fewer than 2000 cells available per well [143].

There are several points that have to be considered when interpreting the results of an ATP assay, as several factors are known to affect ATP levels in cells. These include cellular factors such as age, density and growth of the cell population as well as the stage of cell division and any changes to the growth rate. Moreover, environmental factors such as nutrient availability, any changes in oxygen tension, temperature and pressure, the presence of heavy metals and radiation, as well as mutagens could also affect the results of the ATP assay [142, 143].

Finally, laboratory equipment used for the assay could also influence the results. For instance, the type of microtiter plate must be carefully considered as cross-talk (spill-over of light) between neighbouring wells has to be kept to the minimum.

2.2 Aims

This study uses the ATP luminescence-based assay to measure chemosensitivity of primary glioma cultures.

tumours were used. These were Temozolomide (Temozolomide), Carmustine (BCNU) and Cisplatin (Cis).

The aim of this study was to investigate whether these drugs had a significant effect on primary glioma cultures. Furthermore, in addition to the therapeutic dose of each drug, a ten-fold increase and decrease of this concentration was tested for kill efficacy. The obtained results were then analysed for statistical difference and also used for the calculation of the lethal dose 50 (LD50) of each drug.

An additional aim of this work was to determine whether age or gender play a significant role in chemosensitivity testing.

Finally, chemosensitivity testing was evaluated as a potential tool, which could be used in clinical practice. This was done by comparing the obtained chemosensitivity results with the available survival data from donor patients. This way, comparisons could be made between *in vitro* and *in vivo* data allowing evaluation of its potential contribution to clinical practice.

2.3 Materials and methods

2.3.1 Ethical approval

A protocol for obtaining tumour tissue from patients, along with background information for the project to be undertaken and consent forms for patients and next of kin (appendix 1) were drawn up and ethical approval was received from the Cambridgeshire 3 Research Ethics Committee at the National Research Ethics Service. Consent was obtained on most occasions by the lead researcher (E Lekka), but also by other trained Neurosurgeons and clinical nurse practitioners. The patients were informed about using tumour tissue surplus to diagnostic means for purposes of laboratory culture and drug testing. If the patient was unable to consent, a separate form was used to obtain assent from the next of kin. If there was no surplus tumour tissue, then the biopsied tissue was used solely for diagnostic purposes. It was stipulated that no additional surgical procedure would be performed solely for purposes of obtaining tissue for research. The decision about how much surplus tissue was present was made by a consultant Neuropathologist, who also performed the tumour diagnosis. The patients / relatives were also given an information leaflet explaining details of the research and its non-interventional nature (appendix 1). As all the primary culture work was done in a hospital setting, disposal of waste material / tissue was done in a standard fashion as clinical waste and was incinerated. Ethical permission was also obtained form the ethics committee of the University of Central Lancashire to perform sensitivity studies on the cultured primary cells and their subsequent disposal.

2.3.2 Chemosensitivity

Primary tissue cultures were successfully grown from 12 patients. These were treated with Temozolomide, Carmustine and Cisplatin at three different concentrations (therapeutic concentration, 10-fold higher and 10-fold lower). The therapeutic concentrations used have been reported in the literature [144, 145]. Detergent is highly cytotoxic. As a result minimal levels of luminescence are detected. Cells treated with detergent were used as positive controls. Negative controls were cells left untreated. Controls were used in order to establish the baseline and maximum values for the experiments. The measurements were obtained using the CellTiter-GloTM Luminescent Cell Viability Assay made by Promega according to manufacturer's recommendations. The amount of luminescence generated is directly proportional to the ATP content, which reveals metabolically active cells.

2.3.3 Primary glial culture

Fresh tumour tissue was obtained in the operating theatre from 52 patients. The tissue was collected either by image-guided biopsy or by debulking surgery. Once collected, the tissue was immediately put into "Collection medium" (10 ml Penicillin and Streptomycin solution, 5 ml Amphotericin B solution added to 500 ml of DMEM) and was brought to the laboratory where it was handled under a class III ventilation hood. Forty-nine specimens were processed immediately, while the remaining three were stored at 4°C for less than 24 hours. A consultant Neuropathologist handled the specimen with flamed forceps and sterile scalpel. After transferring the tissue to a petri dish, some fresh collection medium was added and obvious non-tumorous or necrotic material was removed. Initially sufficient tumour tissue was removed for diagnostic purposes. All specimens had tissue confirmation of malignant glioma by smear preparation prior to entering our study. The tumour fragments were then transferred to a fresh petri dish with fresh Collection Medium and diced into small pieces using sterile crossed scalpels. These were then transferred to a 15

ml sterile centrifuge tube containing 10-15 ml Collection Medium. Larger pieces were allowed to settle and the supernatant was discarded. This process was repeated twice in order to remove necrotic, non-viable material.

The tumour fragments were then resuspended in 10 ml Complete Growth Medium in 25 cm^3 cell culture flask and 1 ml of collagenase solution was added. The flask was placed in the incubator for 4 hours at 37^oC. The flask was mechanically agitated every 15 minutes until the tissue was reduced to small cell aggregates. The monitoring of the disaggregation was done with periodic inspection under an inverted microscope.

The preparation was then pipetted several times to promote further disaggregation, then centrifuged at 1000 rpm (250g) for 5 minutes and the enzyme solution was discarded.

The cells were finally washed in 10 ml Complete Growth Medium, then centrifuged again at 1000 rpm (250g) for 5 minutes, discarding the supernatant. The cells were then resuspended in 10 ml of Complete Growth Medium and finally transferred to a 25cm^3 cell culture flask leaving them to incubate at 37^oC.

After 72 hours of incubation, the medium and non-adherent material was discarded. The culture was refed with fresh Complete Growth Medium and returned to the incubator.

The culture was examined twice a week under the inverted microscope and refed as required. Once confluence was achieved, the culture was trypsinised and used for Chemosensitivity testing or divided into two flasks to promote further growth.

The Growth medium used was Ham's F10 nutrient mixture (Sigma Aldrich, cat. no. N2147, LOT no: 018K2343, RNBB0737 and RNBB3121) and DMEM (Dulbecco's Modified Eagle's Medium) in a 1:1 ratio, with 10% foetal calf serum, and 0.5% penicillin / streptomycin and 1% L-glutamine (when not premixed in the medium), sterile filtered. The full composition of Ham's F10 and DMEM is given in appendix 2. The growing cultures were regularly inspected, and media changed when the colour became orange to yellow, and trypsinised when confluence was achieved. Up to half the medium from culture flasks from cultures that grew well was filtered (0.2 μm) and stored as conditioned

medium. This conditioned medium was used to augment growth of slower growing cultures.

2.3.4 Passaging cell cultures

Spent medium was removed from the culture flask by aspiration and the flask was then washed twice with 10ml of Hank's balanced salt solution (HBSS). One ml of 0.25% Trypsin/EDTA solution (stored as 5 ml aliquots at -20°C) was then pipetted into the flask. The flask was left to incubate for 2-10 minutes until the cells began to detach. This process was confirmed by observing at intervals under an inverted microscope. Two milliliters of Complete Growth Medium was then added to the flask to inactivate the trypsin and the cells were pipetted to resuspend and to break down any large cell aggregates. The trypsinised cells were counted in a cell coulter counter, and further dilutions performed if necessary. Depending on the cell density, the total volume of the medium in the flask was then split into two or three new flasks adding fresh growth medium. These were then placed in a CO_2 incubator at 37°C to continue cell growth. The volume of medium required to be added was calculated based on the number of tests to be performed, and the number of cells per ml of fluid required. For the ATP assay, 96-well white microtiter plates with micro clear bottoms (Nunc or Grenier) were used.

2.3.5 Cell counting method using the coulter counter

After switching on the Coulter Particle Counter (Beckman Coulter Z1), the aperture tube was flushed using 10 ml of ISOTON II diluent. The parameters on the SETUP screen were set as: 100µm C, Kd= 58 and Select units <µm> Set lower size: 10 µm. On the OUTPUT screen the result type was set as <concentration> and the dilution factor as 1E+02. The first count was used to calculate the background particle count. The fluid used was ISOTON II diluent only. The result displayed was the number of particles above 10 µm in size per ml of diluent. A hundred microliters of cell suspension was then pipetted into an

Accuvette II vial and 10 ml of ISOTON II diluent was added. Each count was repeated three times and the average was calculated. After completing all cell counts the diluent solutions were replaced with distilled water and the coulter counter switched off.

2.3.6 Making up media

All media, antibiotic solutions and cell culture supplements were commercially obtained from Sigma-Aldrich.

Biopsy Collection Medium

Ten milliliters of Penicillin or Streptomycin solution and 5 ml of Amphotericin B solution were added to 500 ml of a 1:1 mixture of DMEM and Ham's F-12. This was aliquoted in to 15 ml in 30 ml Universal containers (sterile CSF bottles) and stored at 4°C.

Complete Growth Medium

Twelve and a half milliliters of L-Glutamine solution, 5 ml Penicillin or Streptomycin solution and 55 ml Fetal Calf Serum were added to 1000 ml of a 1:1 mixture of DMEM and Ham's F-12. Growth media for every primary culture was kept separate in labeled bottles and stored at 4°C.

Collagenase Solution

Five hundred milligrams of Collagenase Type 1A was dissolved in 31.25 ml of balanced salt solution and aliquoted into 1 ml lots. These were stored at –20°C. The concentration of collagenase achieved was 2000 digestion units per ml.

2.3.7 Defining passages

A passage was defined as every trypsinisation performed in order to detach adherent cells. The first trypsinisation was performed after confluence has been achieved; subsequent to disaggregation and plating of the tumour tissue was defined as passage 0. This was always performed at confluence. Every subsequent trypsinisation was given an increasing number (i.e. passage 1, passage 2, passage 3). Occasionally in order to increase cell density, trypsinisation was performed at sub confluence, by putting cells in smaller flasks, or concentrating cells from three flasks into one. The proliferative capacity of the cells was not reflected into the time to passage in these cases.

2.3.8 Preparation of chemotherapeutic drugs

Temozolomide

Temozolomide (100 mg) was dissolved in 200ml growth medium, aliquoted into 200 aliquots of 1ml and stored at –20°C. To make Stock 1 solution 900µl of growth medium was added to 100µl storage solution (dilution 500µg in 10ml, or 50µg in 1ml). To make Stock 2 solution 900µl of growth medium was added to 100µl of Stock 1 solution (Dilution 50µg in 10ml, or 5µg in 1ml). The volumes and final concentrations in a 96 well plate were as in the table below (Table 2-1):

Table 2-1 ***Temozolomide final dilutions and volumes required for carrying out the ATP assay.***

	Stock 2	Stock 1	Storage sol
Final Dilutions (µg / ml)	2	20	200
Vol of Cell Suspension (µl)	200	200	200
Vol of Growth Medium (µl)	120	120	120
Vol of stock solution (µl)	80	80	80

BCNU (Carmustine)

Carmustine (100 mg) was dissolved in 40 ml of growth medium and aliquoted into 200 aliquots of 1 ml. The aliquots of 200µl with dilution of 2500µg in 1ml were stored at –20°C. To make Stock 1 solution 1800µl of growth medium was added to the one aliquot of the above storage solution (dilution 250µg in 1ml). To make Stock 2 solution 900µl of growth medium was added to 100µl of Stock 1 solution (dilution 25µg in 1ml). To make Stock solution 3, 900µl of growth medium was added to 100µl of Stock solution 2 (dilution 2.5µg in 1ml).
The volumes and final concentrations in a 96 well plate were as in the table below (Table 2-2):

Table 2-2 ***Carmustine final dilutions and volumes required for carrying out the ATP assay.***

	Stock 3	Stock 2	Stock 1
Final Dilutions (µg / ml)	1	10	100
Vol of Cell Suspension (µl)	200	200	200
Vol of Growth Medium (µl)	120	120	120
Vol of stock solution (µl)	80	80	80

Cisplatin

Cisplatin (25 mg) was dissolved in 25ml growth medium, aliquoted into 125 aliquots of 200µl and stored at –20°C (dilution 1000µg in 1ml). To make Stock 1 solution 1800µl of growth medium was added to one aliquot of the above storage solution (dilution 100µg in 1ml). To make Stock 2 solution 900µl of growth medium was added to 100µl of Stock 1 solution (dilution 10µg in 1ml). To make Stock solution 3, 900µl of growth medium was added to 100µl of Stock solution 2 (dilution 1µg in 1ml).The volumes and final concentrations in a 96 well plate were as in the Table 2-3:

Table 2-3 *Cisplatin final dilutions and volumes required for carrying out the ATP assay.*

	Stock 3	Stock 2	Stock 1
Final Dilutions (µg / ml)	0.4	4	40
Vol of Cell Suspension (µl)	200	200	200
Vol of Growth Medium (µl)	120	120	120
Vol of stock solution (µl)	80	80	80

Detergent

Fifty microliters of detergent (Igepal CA-30 from Sigma, product number I-3021) were diluted with 1550µl of complete growth medium. Two hundred microliters were used for each well.

2.3.9 Cell-Titer-Glo Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantisation of the ATP present, which signals the presence of metabolically active cells. The CellTiter-Glo® Assay is designed for use with multiwell-plate formats, making it ideal for automated high-throughput screening (HTS), cell proliferation and cytotoxicity assays. The homogeneous assay procedure involves addition of a single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium (except when a cytotoxic agent interferes with the fluorescent signal) or multiple pipetting steps are not required. Addition of CellTiter-Glo® reagent followed by mixing results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture in agreement with previous reports [146]. The CellTiter-Glo® Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo. Recombinant Luciferase), which in the presence of ATP and molecular oxygen catalyses the mono-oxygenation of luciferin generating

luminescence. The half-life of the produced luminescence is greater than five hours (depending on the cell type and culture medium used) allowing processing of multiple plates. The lyophilized CellTiter-Glo® Substrate and CellTiter-Glo® Buffer should be stored at -20°C. For frequent use, the CellTiter-Glo® Buffer can be stored at 4°C or at room temperature for 48 hours without loss of activity. Reconstituted CellTiter-Glo® Reagent (Buffer plus Substrate) can be stored at 4°C for 48 hours with ~5% loss of activity or at 4°C for 4 days with ~20% loss of activity.

The unique homogeneous format reduces pipetting errors that may be introduced during the multiple steps required by other ATP-measurement methods. The amount of ATP in cells correlates with cell viability. Within minutes after loss of membrane integrity, cells lose the ability to synthesize ATP and endogenous ATPases destroy any remaining ATP. The CellTiter-Glo® Reagent does three things upon addition to cells. It lyses cell membranes to release ATP; it inhibits endogenous ATPases, and it provides luciferin and luciferase necessary to measure ATP using a bioluminescent reaction. The glow type luminescence in this assay is obtained by slow inhibition of the luciferase reaction, while at the same time stabilizing the ATP giving a constant light output, which lasts for 3 – 5 hours (personal communication from Promega).

Assay methodology

Each individual tumour was cultured according to the culture protocol described previously. Confluence was assessed daily under the inverted microscope and when near confluence was achieved the cells were trypsinised (as described in section 2.3.4). One milliliter of trypsin was used in 25 cm^3 flasks, and 3 ml of trypsin was used in 75 cm^3 flasks. The cells were detached from the flask and the trypsin was neutralized with double its volume of growth medium. The cells were thoroughly mixed by pipetting and counted in a coulter counter. The required volume of cell suspension medium was calculated, after taking into account the number of drugs and dilutions to be used for the assay. To the remaining cell suspension further growth medium was then added and

the flask was placed in the incubator. An estimate of the number of cells (initial) per well was calculated. Two hundred microliters of cell suspension was added to each well of a 96-well plate and left for 72 hours in the incubator in order to allow the cells to attach and grow. White plates with clear bottoms (Nunc or Grenier) were used for the assay. After 72 hours, the media was aspirated from each well and replaced immediately with 120 µl of complete growth medium and 80 µl of appropriate drug solutions. Two hundred microliters of detergent solution was added to wells labelled "detergent". Two hundred microliters of complete growth medium was added to wells labelled "media only". The plate was returned to the incubator and left for 48 hours.

The 96-well plate layout was as shown in the table below (Table 2-4):

Table 2-4 ***Layout of the 96-well plate showing the wells used by each of the solutions. Cis: Cisplatin, TMZ: Temozolomide, BCNU Carmustine.***

	1	2	3	4	5	6	7	8	9	10	11	12
A												ATP
B		Cis	Cis	Cis	BCNU	BCNU	BCNU	TMZ	TMZ	TMZ		0.25µg/ml
C		0.4 µg/ml	4 µg/ml	40 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	2 µg/ml	20 µg/ml	200 µg/ml		ATP
D												0.5µg/ml
E												ATP
F		Media					Detergent					1µg/ml
G												ATP
H												2µg/ml

Reagent Preparation

After thawing the CellTiter-Glo® Buffer the buffer and the lyophilized CellTiter-Glo® Substrate were allowed to equilibrate to room temperature prior to use.
The CellTiter-Glo® Buffer volume (10 mls) was added to the CellTiter-Glo® Substrate to reconstitute the enzyme-substrate mixture which formed the CellTiter-Glo® Reagent. The CellTiter-Glo® Reagent was then mixed by gently

vortexing, swirling or by inverting the contents until a homogeneous solution was obtained.

Protocol for carrying out the assay

The multiwell plate and its contents were equilibrated to room temperature for approximately 30 minutes. That was an important step as temperature can affect the rate of reaction of luciferase and therefore the intensity of the signal. Insufficient equilibration could result in a temperature gradient effect between the wells in the centre and on the edge of the plates.

Whilst the plate was equilibrating, the 20 µg/ml stock ATP was serially diluted to make the four required ATP concentrations. Then, 900 µl of PBS were added to 100 µl of 20 µg/ml of ATP to make 1 ml of Stock 1 solution of ATP (dilution of 2µg/ml). To make Stock 2 solution, 500 µl of PBS were added to 500 µl of Stock 1 (dilution of 1µg/ml). To make Stock 3 solution, 500 µl of PBS were added to 500 µl of Stock 2 (dilution of 0.5µg/ml). To make Stock 4 solution, 500 µl of PBS were added to 500 µl of Stock 3 (dilution of 0.25µg/ml).The volumes and final concentrations of ATP are shown in the table below (Table 2-5):

Table 2-5 ***ATP final dilutions and volumes required for carrying out the ATP assay.***

Final Conc of ATP	Vol of ATP	Vol PBS
2 µg/ml Stock 1	100 µl of 20 µg/ml	900 µl
1 µg/ml Stock 2	500 µl of 2 µg/ml	500 µl
0.5 µg/ml Stock 3	500 µl of 1 µg/ml	500 µl
0.25 µg/ml Stock 4	500 µl of 0.5 µg/ml	500 µl

The medium from each well of the multiwell plate was removed and each well was washed with 100 µl of PBS. Then, 100 µl of PBS were added to each well and two replicates of each of the ATP standards were added to the final column (Table 2-4 column 12). An equal volume (100 µl) of CellTiter-Glo® Reagent was added to each well to ensure the correct 1x concentration of the reagent. The

contents were mixed for 2 minutes on an orbital shaker to induce cell lysis. The plate was then incubated at 25°C for 10 minutes to stabilize. Luminescence was measured in a plate reader (Tecan GENios Pro®) (Figure 2-1).

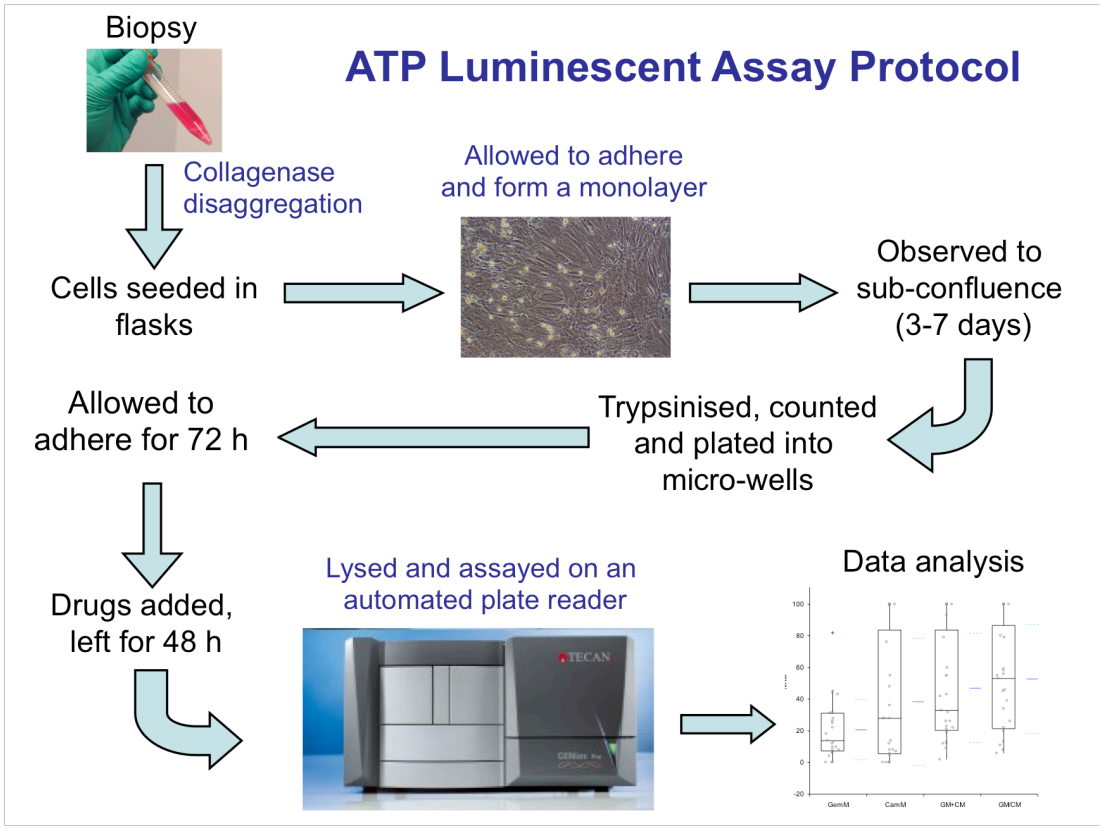

Figure 2-1 ATP Luminescent Assay Protocol.

2.3.10 Lethal Dose 50 (LD50)

The lethal dose (LD) indicates lethality of a substance and is a commonly used concept in chemosensitivity studies. The number that follows the lethal dose indicates the percentage of the kill produced by the substance. For example, LD50 is the drug dose that kills 50% of the treated population. Following this convention, a cell line or primary culture is defined arbitrarily as being chemosensitive after exposure to a drug concentration, when the cells surviving are 50% of the untreated cells. For the ATP assay used in this study, the LD50 is the dose of the drug at which the luminescence measured was half of the one of the untreated cells. The values obtained were in four repeats and the mean of

each drug concentration was plotted on a graph. A linear trendline was then fitted on each plot and used to calculate the LD50.

2.4 Results:

2.4.1 Establishing primary glioma cultures

Fifty-two brain tumour specimens were received during the period of May 2009 to November 2010. Of those, forty-five were successfully cultured whereas the remaining seven did not grow. Thirty-three out of the forty-five successful cultures got contaminated prior to testing and therefore discarded. Contamination is a well-known problem in primary tissue culture. The contamination observed in this study is in line with the contamination levels of reported studies as well as previous studies in our institution. The remaining twelve cultures were taken forward to chemosensitivity testing (Table 2-6).

Table 2-6 Summary of attempted primary glioma cultures.

BTNW NO.	PROGRESS	DRUG TESTING	BTNW NO.	PROGRESS	DRUG TESTING
338	Tested	BCNU, TMZ, Cis	**453**	No growth	
352	Contaminated		**458**	Contaminated	
365	Tested	BCNU, TMZ, Cis	**460**	Contaminated	
370	Tested	BCNU, TMZ, Cis	**462**	Contaminated	
374	Tested	BCNU, TMZ, Cis	**470**	Contaminated	
377	Tested	BCNU, TMZ, Cis	**482**	Contaminated	
382	Tested	BCNU, TMZ, Cis	**484**	Contaminated	
390	Contaminated		**500**	Tested	BCNU, TMZ, Cis
396A	Contaminated		**502**	Contaminated	
396B	Contaminated		**512**	No growth	
404	Contaminated		**515**	No growth	
410	Contaminated		**522**	Contaminated	
411	Contaminated		**533**	Tested	BCNU, TMZ, Cis
412A	Contaminated		**535**	Contaminated	
412B	Contaminated		**538**	Tested	BCNU, TMZ, Cis
416	Contaminated		**544**	Contaminated	
418	Contaminated		**545**	No growth	
421	Contaminated		**546**	Tested	BCNU, TMZ, Cis
428	No growth		**590**	Tested	BCNU, TMZ, Cis
435	Contaminated		**597**	Tested	BCNU, TMZ, Cis
436	Contaminated		**626**	Contaminated	
439	Contaminated		**632**	No growth	
442	Contaminated		**640**	No growth	
445A	Contaminated		**651**	Contaminated	
445B	Contaminated		**676**	Contaminated	
452	Contaminated		**693**	Contaminated	

Summary of attempted primary glioma cultures. Out of fifty-two, seven did not grow, thirty-three got contaminated and twelve were successfully tested. BCNU Carmustine, TMZ Temozolomide, Cis Cisplatin.

2.4.2 Chemo-sensitivity Analysis

To compare the effect of the drug dosage on the kill efficiency of the drugs tested, the nonparametric Kolmogorov-Smirnov statistical test (KS test) was used [147]. The Kolmogorov–Smirnov statistic quantifies the distance between the empirical distribution functions of two samples. In this case, the cumulative distribution of the cells from each concentration of each drug was pair-wise compared against the cumulative distribution of each of the two control cell cultures (those containing only the growth medium and those with the detergent). For a drug to be effective at a given concentration it needs to exhibit kill efficiency greater than the one observed when only the control growth medium is used. Moreover, the higher the kill efficiency of the drug, the closer to the kill efficiency observed by the detergent it would be. The null hypothesis in this case suggests that the drugs tested are not significantly different from each of the two controls. In terms of evaluating statistical significance given a calculated p-value, both significance levels α=0.001 (conservative) and α=0.05 (widely used) were considered. If a test of significance gives a p-value lower than the significance level α, the null hypothesis is rejected and the sample can be considered as being statistically significantly different from the control it was compared against.

2.4.2.1 Statistical package

The statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.15.1) running under Windows Vista OS with 2GB RAM [148]. All the statistical tests used here were included in the R Stats package.

Comparison of drug concentrations against the two controls

In this section, three concentrations (100x, 10x, and 1x where 10x being the therapeutic dose) from each tested drug (Cisplatin, Carmustine and Temozolomide) were compared against the kill efficiency of both the growth

medium and the detergent. The results of the comparison are shown on Table 2-7 and Figure 2-2.

Table 2-7 Comparison of the kill efficiency of each of the three drugs to the growth medium and the detergent.

Drugs tested (All Ages and Genders)	**Medium**		**Detergent**	
	Proximity to Medium (KS Distance)	**p-value**	**Proximity to Detergent (KS Distance)**	**p-value**
Cis100	0.521	**<0.001**	0.917	**<0.001**
Cis10	0.271	0.059	0.917	**<0.001**
Cis1	0.167	0.522	0.938	**<0.001**
Carmu100	0.188	0.368	0.917	**<0.001**
Carmu10	0.188	0.368	0.938	**<0.001**
Carmu1	0.167	0.522	0.938	**<0.001**
TMZ100	0.313	**<0.001**	0.958	**<0.001**
TMZ10	0.229	0.161	0.979	**<0.001**
TMZ1	0.167	0.522E	0.958	**<0.001**

Comparison of the kill efficiency of three concentrations of Cisplatin (Cis100, Cis10, Cis1), Carmustine (Carmu100, Carmu10, Carmu1) and Temozolomide (TMZ100, TMZ10, TMZ1) against that of the growth medium and the detergent. Significant values are shown in bold.

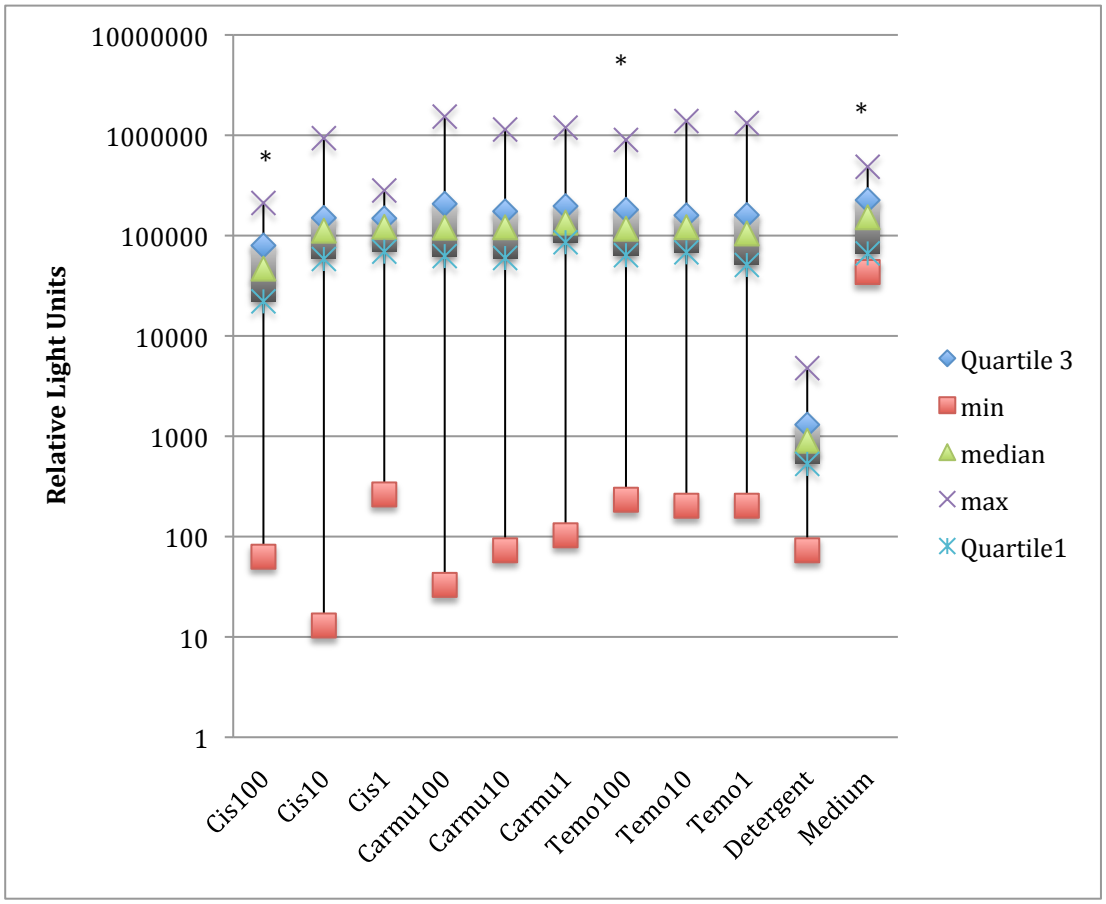

Figure 2-2 Boxplot summarizing the Relative Light Units (RLU) measured in cultures of all patients tested with Cisplatin (Cis), Carmustine (Carmu), Temozolomide (TMZ) in three concentrations (x100, x10, and x1). RLU of the untreated cultures (Medium) and those treated with Detergent are also shown. Asterisk (*) indicates statistical significance between datasets with P<0.05.

Based on Table 2-7, all three tested concentrations from all three tested drugs were not found to be as effective as the detergent (α=0.05). More importantly, only Cisplatin 100x and Temozolomide 100x seemed to exhibit a significantly different kill efficiency to the one observed by the growth medium (Cis100: P<0.001; TMZ100: P<0.05). Cisplatin 10x was narrowly excluded from having a significant effect when compared to the Medium as the correspondent p-value was marginally higher than our predefined 5% significance level cut off (0.05915). None of the remaining tested drugs and concentrations had shown any significant kill effect in this study.

Drug kill efficiency across different age groups

In order to determine whether age plays a role on the kill efficiency of the drug the results were reanalysed after grouping them into two age groups (over 65 and under 65 years of age). The results of this analysis are shown in Table 2-8 and Table 2-9 and Figure 2-3 and Figure 2-4.

Table 2-8 Comparison of the kill efficiency of each drug to the growth medium in the over 65 years age group.

Drugs used (Age >65, All Genders)	Proximity to Medium (KS Distance)	p-value
Cis100	0.464	**<0.05**
Cis10	0.321	0.111
Cis1	0.179	0.773
Carmu100	0.179	0.763
Carmu10	0.179	0.773
Carmu1	0.214	0.549
TMZ100	0.429	**<0.05**
TMZ10	0.321	0.111
TMZ1	0.179	0.773

Comparison of the kill efficiency of three concentrations of Cisplatin (Cis100, Cis10, Cis1), Carmustine (Carmu100, Carmu10, Carmu1) and Temozolomide (TMZ100, TMZ10, TMZ1) against that of the growth medium in both genders for the over 65 years age group. Significant values are shown in bold.

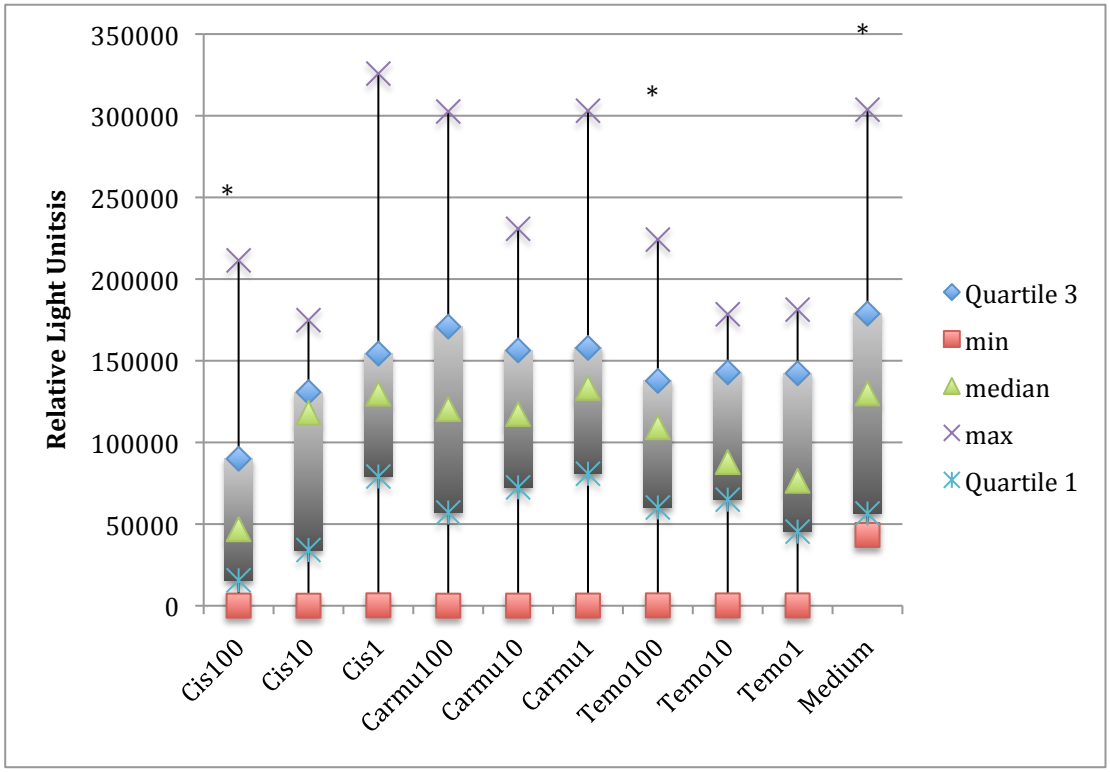

Figure 2-3 Boxplot summarizing the Relative Light Units (RLU) measured in cultures of all patients over 65 years of age tested with Cisplatin (Cis), Carmustine (Carmu), Temozolomide (TMZ) in three concentrations (x100, x10, and x1) and the untreated. Asterisk (*) indicates statistical significance between datasets with P<0.05.

According to Table 2-8, for the over 65 years age group, only Cisplatin 100x and Temozolomide 100x showed a significant effect when compared to the control (growth medium) (α=0.05). None of the remaining drugs and concentrations had shown any significant kill effect (α=0.05).

Table 2-9 Comparison of the kill efficiency of each of the three drugs to the growth medium in the under 65 years age group

Drugs used (Age <65, All Genders)	Proximity to Medium (KS Distance)	p-value
Cis100	0.800	**<0.001**
Cis10	0.350	0.173
Cis1	0.400	0.081
Carmu100	0.400	0.081
Carmu10	0.400	0.081
Carmu1	0.400	0.081
TMZ100	0.250	0.571
TMZ10	0.400	0.081
TMZ1	0.400	0.081

Comparison of the kill efficiency of three concentrations of Cisplatin (Cis100, Cis10, Cis1), Carmustine (Carmu100, Carmu10, Carmu1) and Temozolomide (TMZ100, TMZ10, TMZ1) against that of the growth medium in both genders for the under 65 years age group. Significant values are shown in bold.

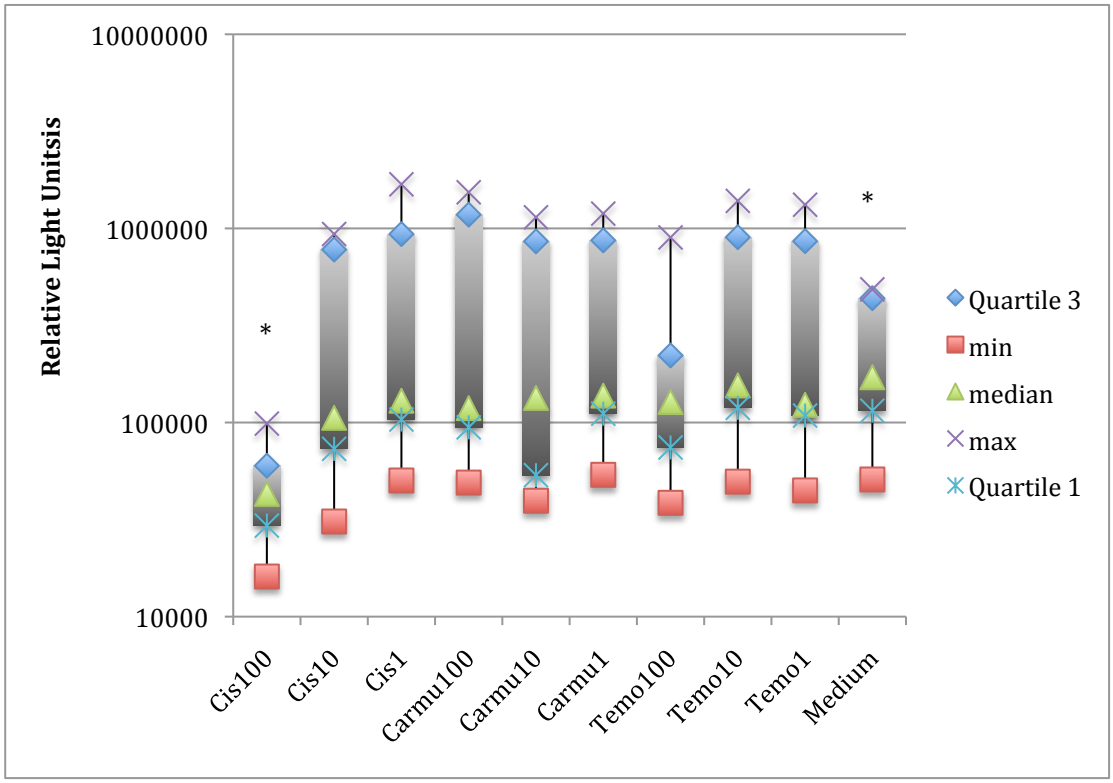

Figure 2-4 Boxplot summarizing the Relative Light Units (RLU) measured in cultures of all patients under the age of 65 years tested with Cisplatin (Cis), Carmustine (Carmu), Temozolomide (TMZ) in

three concentrations (x100, x10, and x1) and the untreated. Asterisk (*) indicates statistical significance between datasets with P<0.001.

In the under 65 years age group, only Cisplatin 100x showed a significant effect when compared to the Control (growth medium) but this time with a higher confidence score (α=0.001). None of the remaining drugs and concentrations had shown any significant kill effect (α=0.05).

Comparing Table 2-8 and Table 2-9, the age group of under 65 years exhibits a better response to the drugs as their KS Distance values were consistently equal or greater than those of the over 65 years age group. In addition, the p-values of the under 65 years age group where overall lower than those of the over 65 years age group and in some cases not too distant from the significance cut-off value of 0.05 (0.08106). This suggests that the under 65 years age group might respond better to chemotherapy treatment with the aforementioned drugs although this is just a comparison of the significance of the observed results and should be treated with caution.

Drug kill efficiency in relation to gender

In order to determine whether gender plays a role on the kill efficiency of the drug, the datasets were reanalysed after being divided into male and female groups. The results of this analysis are shown in Table 2-10 and Table 2-11 and Figure 2-5 and Figure 2-6.

Table 2-10 Comparison of the kill efficiency of each drug for female patients from both age groups

Drug tested (Female, All Ages)	Proximity to Medium (KS Distance)	p-value
Cis100	0.714	**<0.0001**
Cis10	0.429	**<0.05**
Cis1	0.286	0.205
Carmu100	0.321	0.111
Carmu10	0.286	0.205
Carmu1	0.286	0.205
TMZ100	0.393	**<0.05**
TMZ10	0.286	0.205
TMZ1	0.286	0.205

Comparison of the kill efficiency of three concentrations of Cisplatin (Cis100, Cis10, Cis1), Carmustine (Carmu100, Carmu10, Carmu1) and Temozolomide (TMZ100, TMZ10, TMZ1) against that of the growth medium for female patients in both age groups. Significant values are shown in bold.

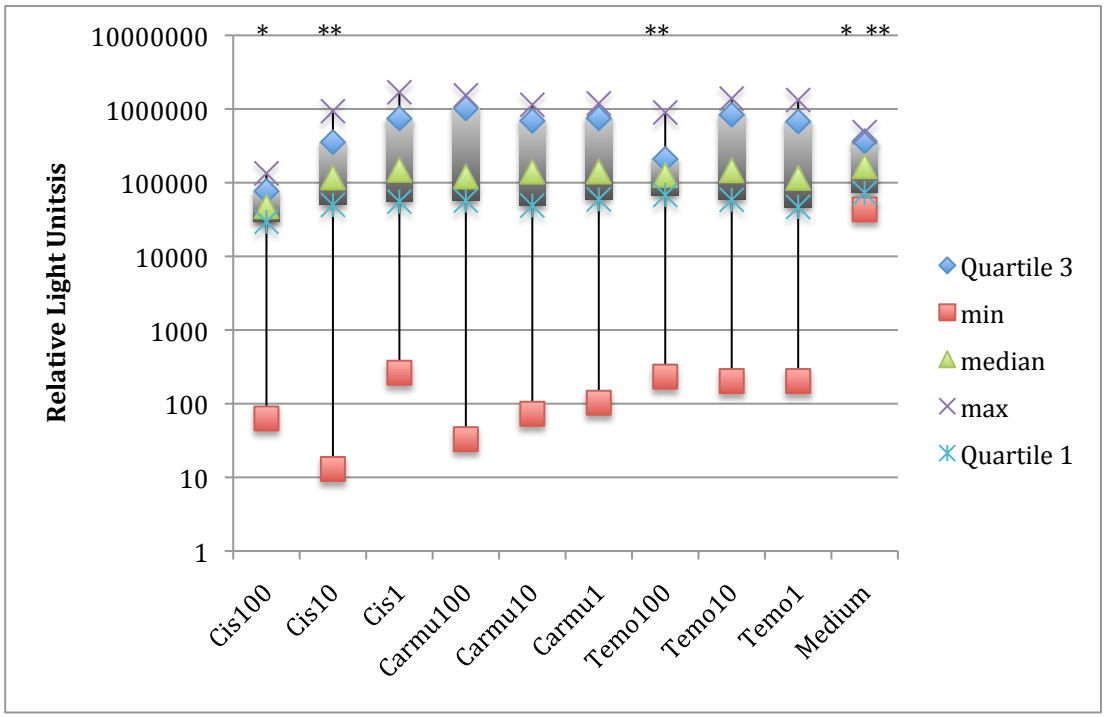

Figure 2-5 Boxplot summarizing the Relative Light Units (RLU) measured in cultures of female patients of all ages tested with Cisplatin (Cis), Carmustine (Carmu), Temozolomide (TMZ) in three

concentrations (x100, x10, and x1) and the untreated. Asterisks (*) indicates statistical significance between datasets with * P<0.001 and ** P<0.05.

Based on these results, Cisplatin 100x exhibited a significantly different kill efficiency to the one observed by the growth medium (α=0.001). Cisplatin 10x and Temozolomide 100x were also had a significant effect at the less conservative cut off value of α=0.05. None of the remaining tested drugs and concentrations had shown any significant kill effect.

Table 2-11 Comparison of the kill efficiency of each drug for male patients from both age groups.

Drug tested (Male, All Ages)	Proximity to Medium (KS Distance)	p-value
Cis100	0.600	**<0.001**
Cis10	0.200	0.819
Cis1	0.250	0.571
Carmu100	0.200	0.832
Carmu10	0.200	0.832
Carmu1	0.300	0.336
TMZ100	0.250	0.571
TMZ10	0.250	0.571
TMZ1	0.250	0.571

Comparison of the kill efficiency of three concentrations of Cisplatin (Cis100, Cis10, Cis1), Carmustine (Carmu100, Carmu10, Carmu1) and Temozolomide (TMZ100, TMZ10, TMZ1) against that of the growth medium for male patients in both age groups. Significant values are shown in bold.

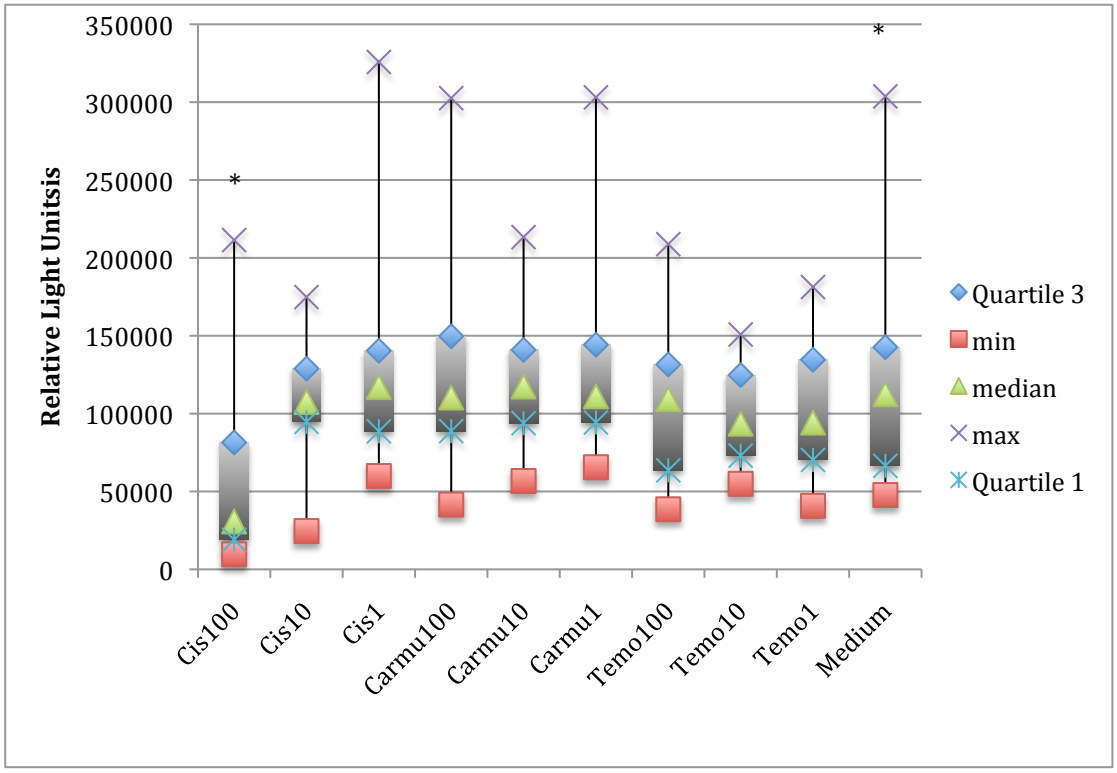

Figure 2-6 Boxplot summarizing the Relative Light Units (RLU) measured in cultures of male patients tested with Cisplatin (Cis), Carmustine (Carmu), Temozolomide (TMZ) in three concentrations (x100, x10, and x1) and the untreated. Asterisk (*) indicates statistical significance between datasets with P<0.001.

Similar to Table 2-10, Cisplatin 100x was again significantly different to the control growth medium but only at the less conservative cut off value of α=0.05 as it was slightly higher than α=0.001 (0.00112). None of the remaining tested drugs and concentrations had shown any significant kill effect when compared to the control (α=0.05).

Given these results, females were more sensitive to the tested drugs at the different doses than the males as their ks-distances were generally higher than those of the males (with the exception of Carmustine 1x). Moreover, both Cisplatin 10x and Temozolomide 100x in females were found to be significantly different to the control (α=0.05).

2.4.3 Clinical profile and chemosensitivity results of each individual patient

BTNW 338

Clinical History: This 71-year-old male presented with self-limiting episodes of generalised tonic-clonic seizures. On examination he had a GCS of 14/15 with receptive and expressive dysphasia and right upper limb power of 4+/5. He did not receive any adjuvant treatment and died after 5 weeks.

Radiology: CT scan revealed a left temporal lesion.

Surgery: Craniotomy and debulking performed on the 1st of May 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 24 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to all drugs used (Table 2-12 and Figure 2-7). Given the observed dose response trends Cisplatin was expected to reach LD50 at 55.67 µg/ml.

Table 2-12 Chemosensitivity results for the tested drug.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Resistant (55.67)
Carmustine	Resistant
Temozolomide	Resistant

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

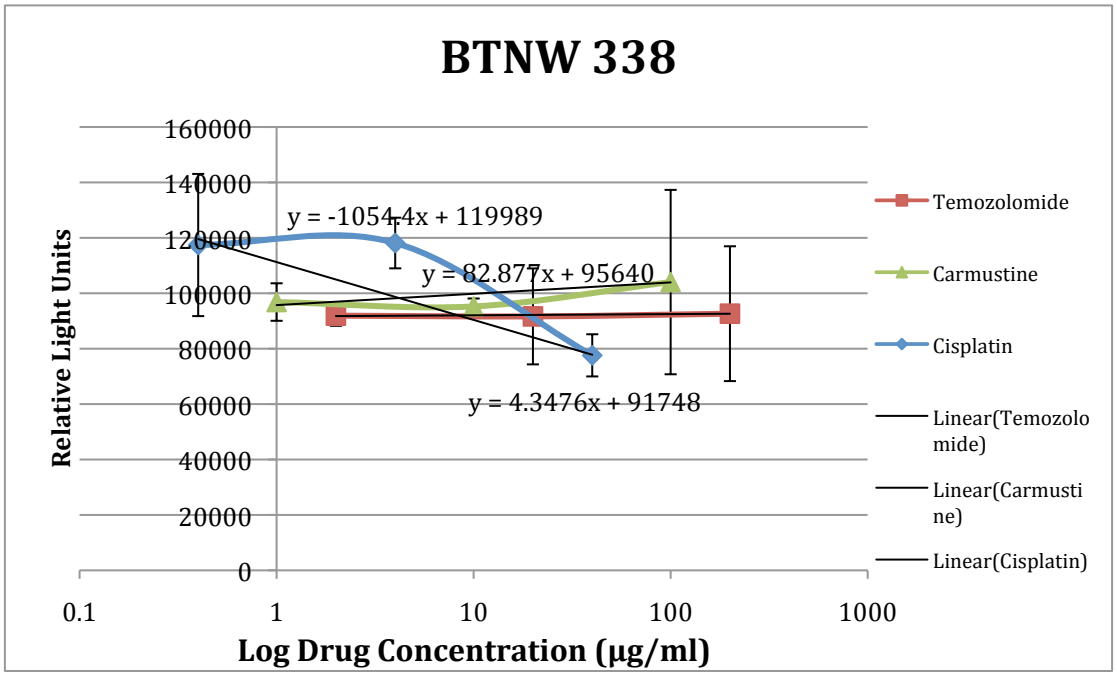

Figure 2-7 ***Dose response curves for BTNW 338 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.***

BTNW 365

Clinical History: This 42-year-old female presented with one episode of generalised tonic-clonic seizures, sleeplessness and increased anxiety for two days. On examination she had a GCS of 15/15 with no focal neurological findings. She received adjuvant Radiotherapy. She died after 163 weeks.

Radiology: CT scan revealed a right fronto-parietal lesion.

Surgery: Craniotomy and debulking performed on the 30th of June 2009.

Histology: Histopathology revealed a WHO grade II Fibrillary Astrocytoma with atypical features. It was felt that overall "sampling" was under-grading this tumour.

Primary culture: Culture became confluent after 20 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-13 and Figure 2-8). Given the observed dose response trends Temozolomide was expected to reach LD50 at 228.04 µg/ml.

Table 2-13 Chemosensitivity results for the tested drugs

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (32.57)
Carmustine	Resistant
Temozolomide	Resistant (228.04)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

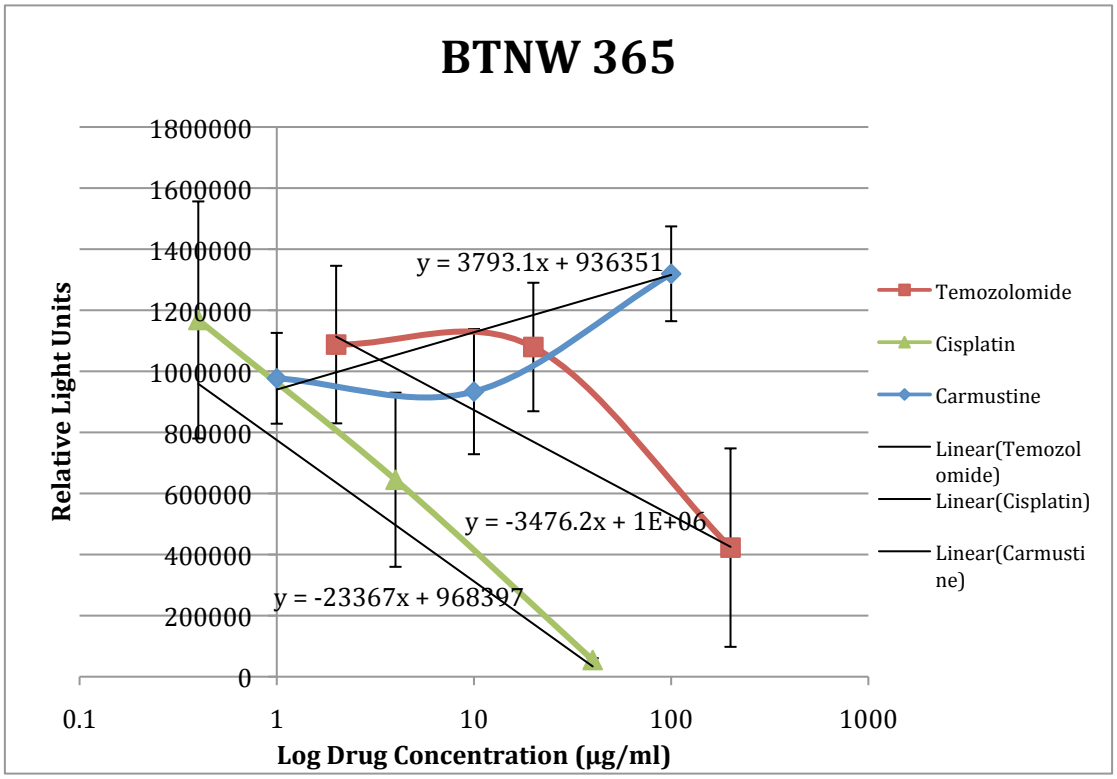

Figure 2-8 Dose response curves for BTNW 365 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 374

Clinical History: This 34-year-old female presented with a four-week history of malaise, nausea and vertigo. On examination she had a GCS of 15/15 with cerebellar signs (nystagmus and past-pointing) as well as visual disturbance. She received adjuvant Radiotherapy (54 Gys) with concomitant Temozolomide chemotherapy. She died after 30 weeks.

Radiology: CT scan was initially thought to be normal, but when an MR was performed, it revealed a posterior fossa lesion.

Surgery: Posterior fossa craniotomy and debulking performed on the 17th of July 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 5 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-14 and Figure 2-9). Given the observed dose response trends Temozolomide was expected to reach LD50 at 209.33 µg/ml.

Table 2-14 Chemosensitivity results for the tested drugs

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (33.50)
Carmustine	Resistant
Temozolomide	Resistant (209.33)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

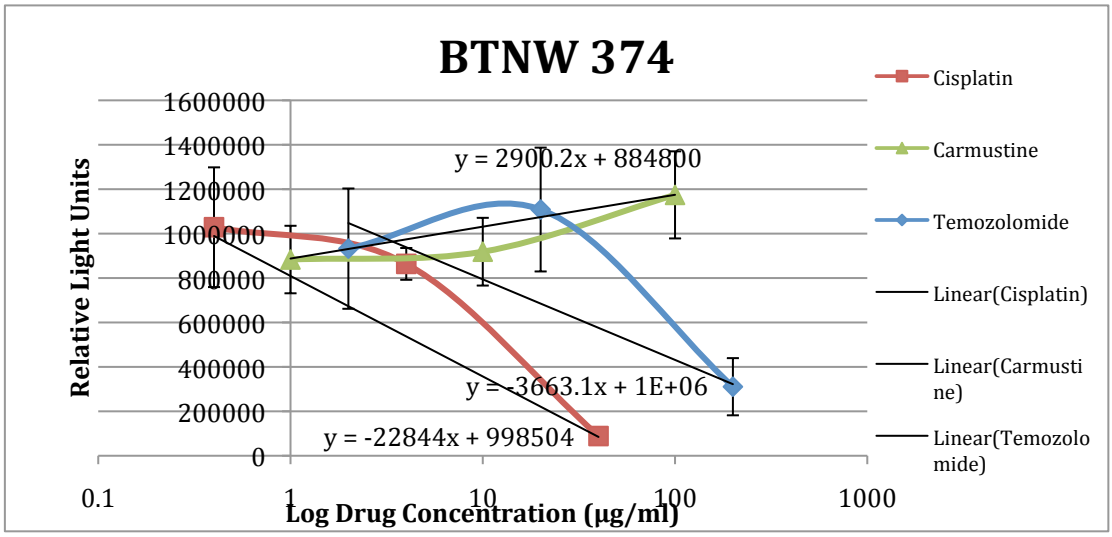

Figure 2-9 Dose response curves for BTNW 374 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 544

Clinical History: This 68-year-old male presented with aphasia, which resolved on steroids. On examination he had a GCS of 15/15 and normal limb power. He received adjuvant Radiotherapy (54 Gys) with concomitant Temozolomide chemotherapy. He died after 89 weeks.

Radiology: CT scan revealed a left fronto-parietal lesion.

Surgery: Craniotomy and debulking performed on the 25th of November 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 56 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-15 and Figure 2-10).

Table 2-15 Chemosensitivity results for the tested drugs

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (37.54)
Carmustine	Resistant
Temozolomide	Resistant

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

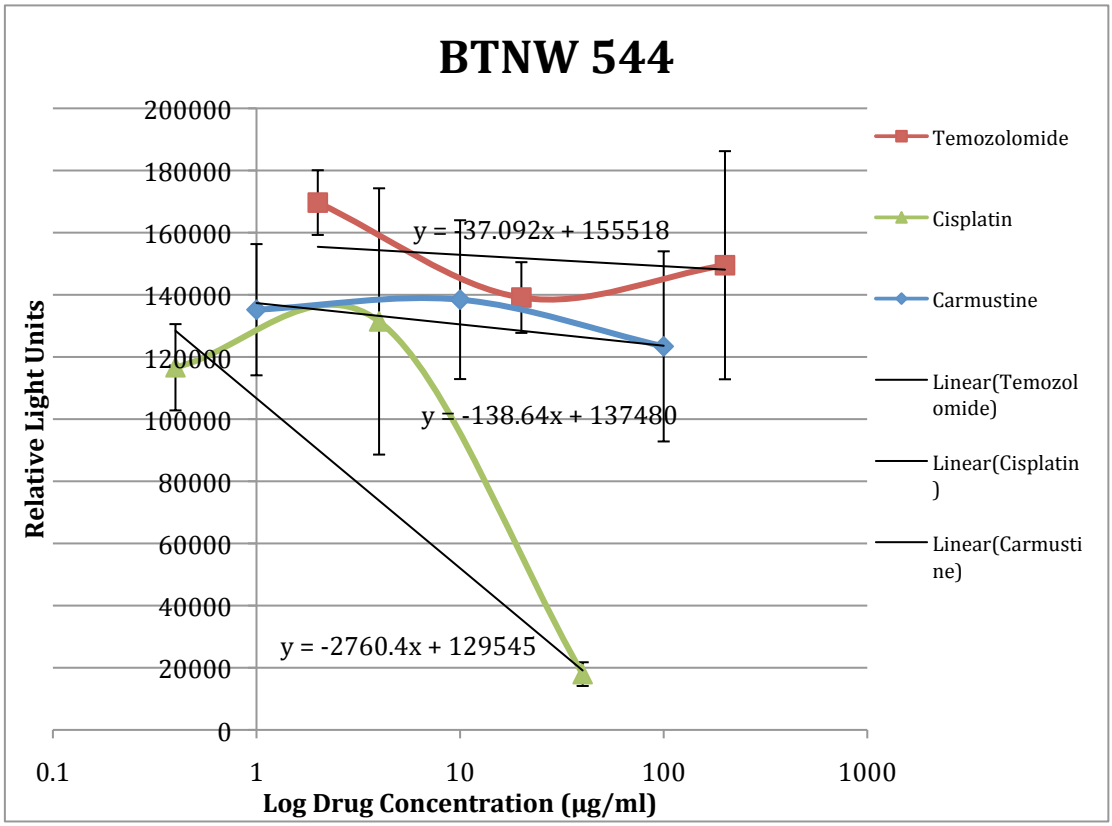

Figure 2-10 Dose response curves for BTNW 544 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 370

Clinical History: This 66-year-old female presented with a three-week history of headaches, poor concentration, feeling unwell, confusion and memory loss. On examination she had a GCS of 14/15 with confusion and dysphasia. Limb power was globally reduced to 4/5. She received palliative radiotherapy and died after 6 weeks.

Radiology: CT scan revealed a left frontal lesion crossing the midline along the corpus callosum.

Surgery: Craniotomy and debulking performed on the 13th of July 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV with gliosarcomatous features.

Primary culture: Culture became confluent after 21 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-16 and Figure 2-11).

Table 2-16 Chemosensitivity results for the tested drugs

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (25.96)
Carmustine	Resistant
Temozolomide	Resistant

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

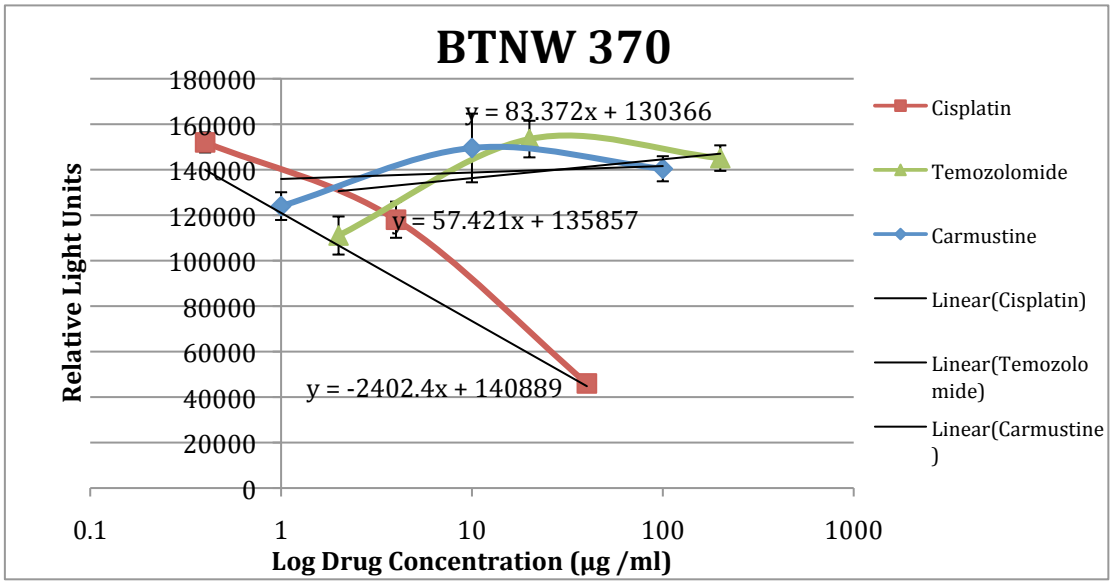

Figure 2-11 Dose response curves for BTNW 370 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 382

Clinical History: This 75-year-old female presented with a six week history of dysphasia and right sided hemiparesis. On examination she had a GCS of 14/15 with receptive and expressive dysphasia and right hemiparesis of 4/5. She received any adjuvant radiotherapy but no chemotherapy treatment. She died after 109 weeks.

Radiology: CT scan revealed a left frontal lesion.

Surgery: Craniotomy and debulking performed on the 04th of August 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 56 days.

Chemosensitivity results: ATP luminescent assay revealed sensitivity to all three drugs tested. (Table 2-17 and Figure 2-12).

Table 2-17 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (<0.4)
Carmustine	Sensitive (< 1)
Temozolomide	Sensitive (64.3)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

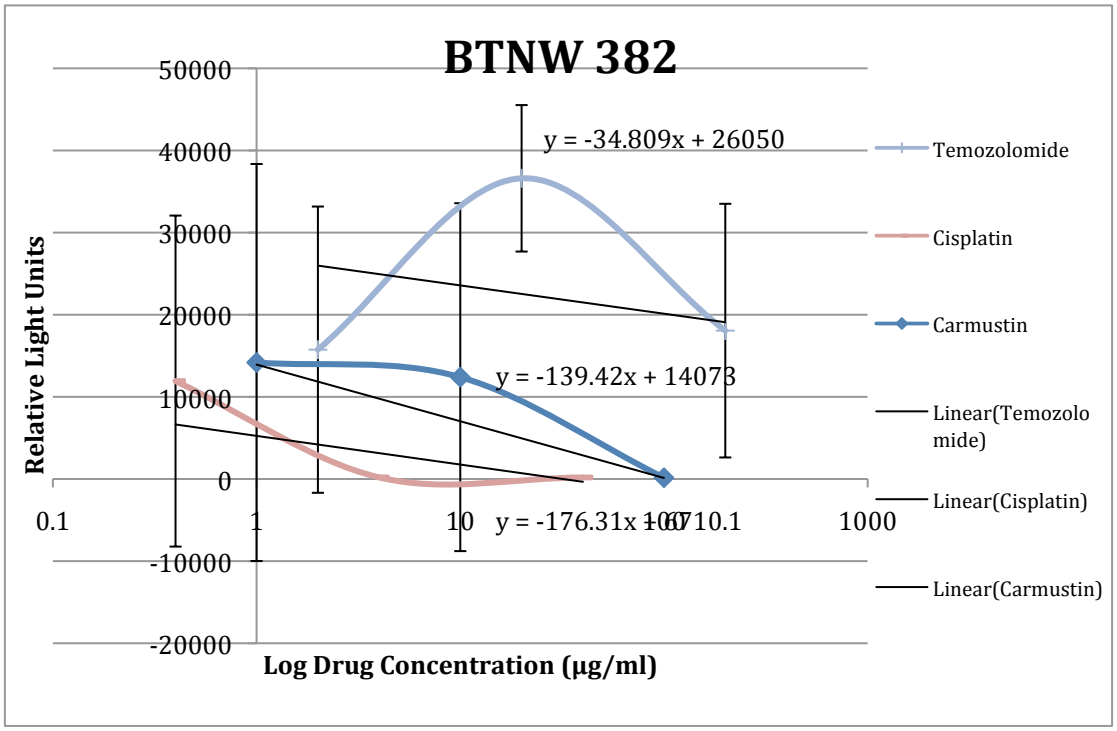

Figure 2-12 Dose response curves for BTNW 382 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 377

Clinical History: This 45-year-old female presented with a four-month history of headaches, confusion and memory loss. On examination he had a GCS of 15/15 and no focal neurological findings. She underwent adjuvant radiotherapy treatment with concomitant Temozolomide chemotherapy. She died after 53 weeks.

Radiology: CT scan revealed a left temporal lesion.

Surgery: Craniotomy and debulking with Gliadel wafers performed on the 22nd of July 2009.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 25 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-18 and Figure 2-13).

Table 2-18 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (32.30)
Carmustine	Resistant
Temozolomide	Resistant

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

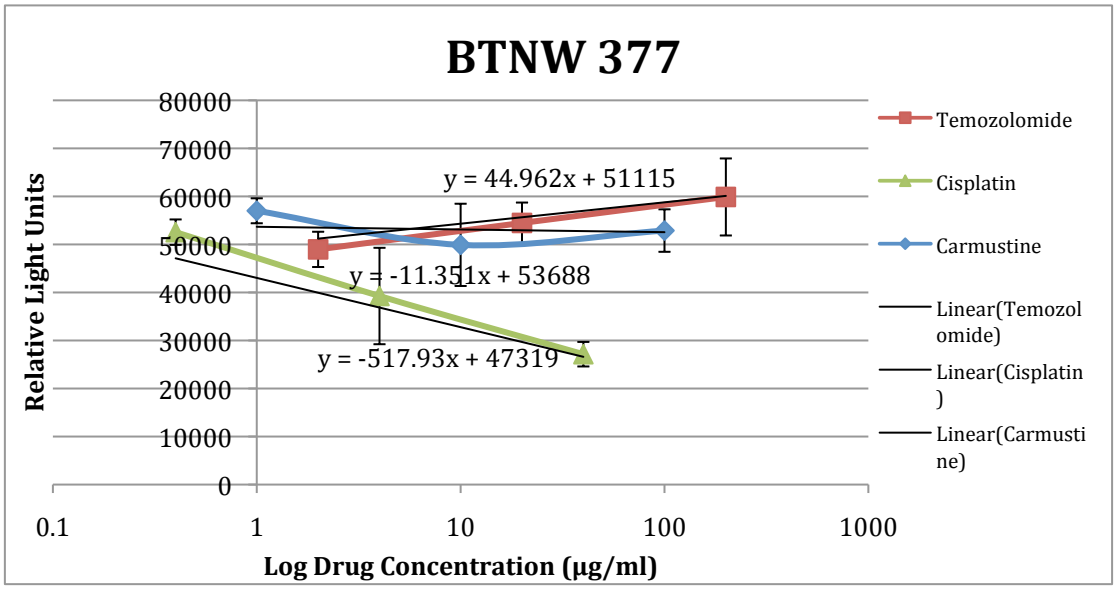

Figure 2-13 Dose response curves for BTNW 377 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 500

Clinical History: This 72-year-old female presented with headaches, memory loss and personality changes. On examination she had a GCS of 15/15 with no focal neurological findings. She received palliative radiotherapy and died after 17 weeks.

Radiology: CT scan revealed a right frontal lesion.

Surgery: Craniotomy and debulking performed on the 23rd of March 2010.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 73 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Cisplatin and Carmustine. Temozolomide reached a 50% cell kill. (Table 2-19 and Figure 2-14). Given the observed dose response trends Cisplatin was expected to reach LD50 at 45.28 µg/ml.

Table 2-19 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Resistant (45.28)
Carmustine	Resistant
Temozolomide	Sensitive (102.7)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

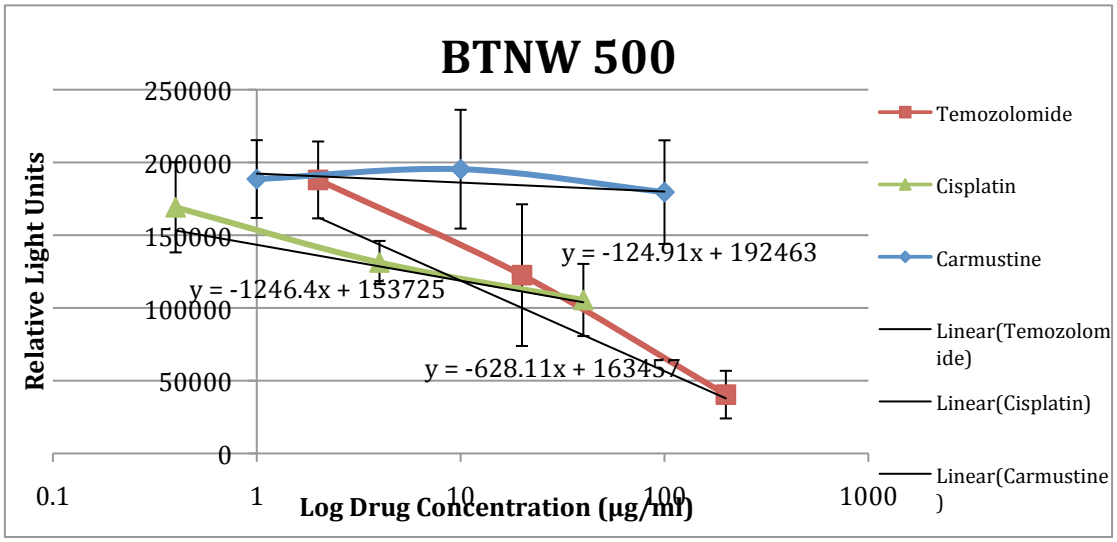

Figure 2-14 Dose response curves for BTNW 500 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 533

Clinical History: This 67-year-old male presented with sudden onset of expressive dysphasia. On examination he had a GCS of 14/15 with receptive and expressive dysphasia and no focal neurological deficit. He received adjuvant radiotherapy (54 Gys) with concomitant Temozolomide treatment and died after 21 weeks.

Radiology: CT scan revealed a left temporal lesion.

Surgery: Craniotomy and debulking performed on the 11th of May 2010.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 24 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Cisplatin and Carmustine. Temozolomide reached a 50% cell kill. (Table 2-20 and Figure 2-15). Given the observed dose response trends Cisplatin was expected to reach LD50 at 82.86 µg/ml.

Table 2-20 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Resistant (82.86)
Carmustine	Resistant
Temozolomide	Sensitive (2)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

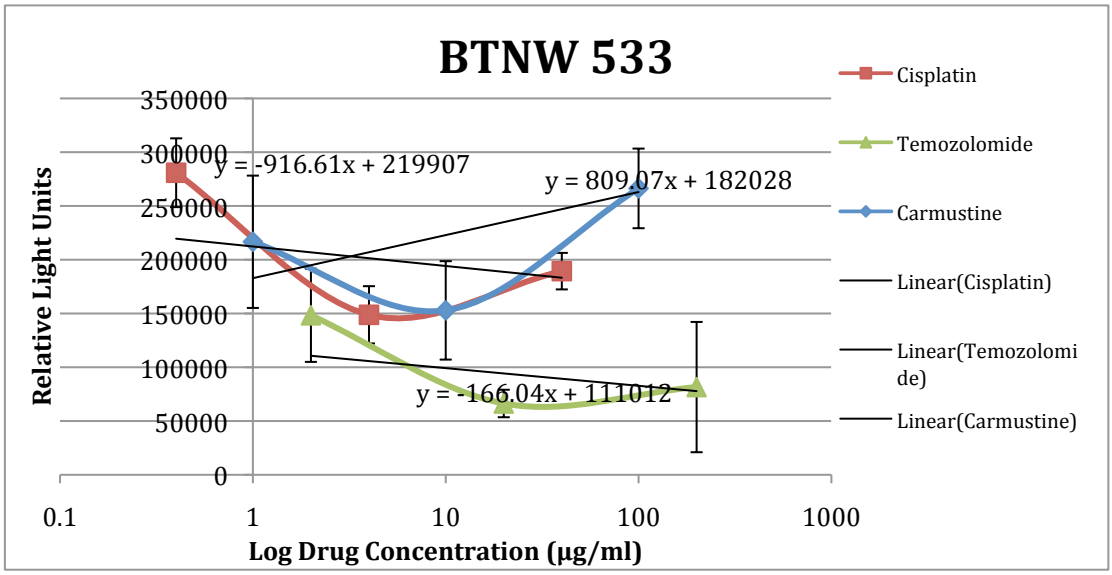

Figure 2-15 Dose response curves for BTNW 533 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 546

Clinical History: This 50-year-old female presented with headache. On examination she had a GCS of 14/15 with receptive and expressive dysphasia and left sided hemiparesis. She did not receive any adjuvant treatment because of her poor Karnofsky score (60). She died after 3 weeks.

Radiology: CT revealed a right frontal intrinsic lesion.

Surgery: Craniotomy and debulking performed on the 25th of May 2010.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 44 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Temozolomide. Cisplatin and Carmustine reached a 50% cell kill. (Table 2-21 and Figure 2-16).

Table 2-21 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (19.29)
Carmustine	Sensitive (7.7)
Temozolomide	Resistant

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

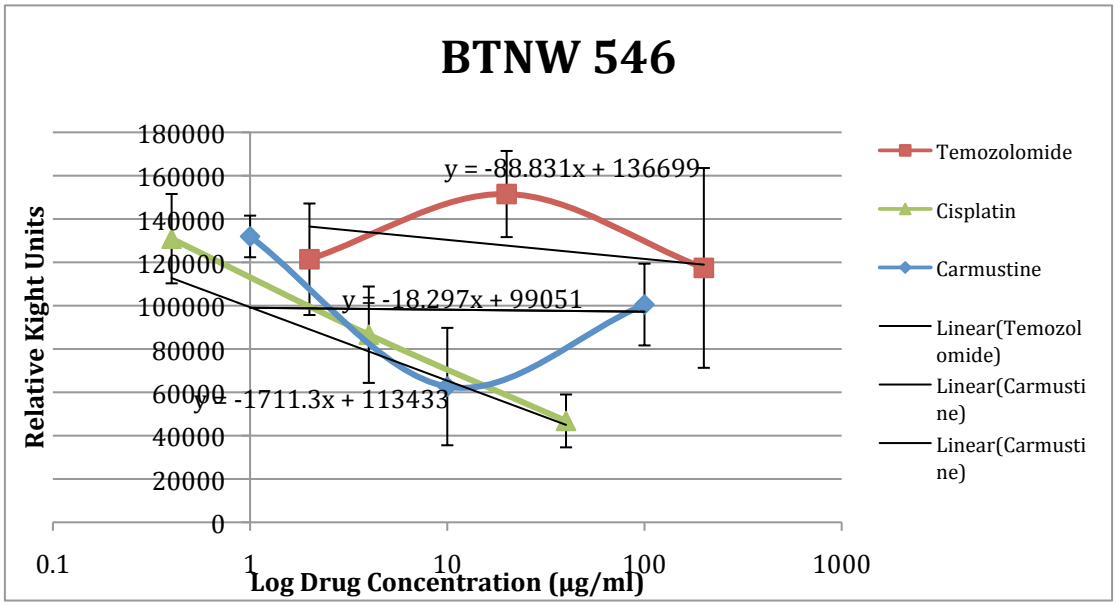

Figure 2-16 Dose response curves for BTNW 546 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 590

Clinical History: This 52-year-old male presented with headaches, expressive dysphasia and right upper limb weakness 4/5. On examination he had a GCS of 15/15 with expressive dysphasia. He received adjuvant radiotherapy (54 Gys) with concomitant Temozolomide followed by adjuvant Temozolomide chemotherapy treatment. He died after 80 weeks.

Radiology: CT revealed a left frontal intrinsic lesion.

Surgery: Craniotomy and debulking performed on the 15th of July 2010.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 203 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-22 and Figure 2-17). Given the observed dose response trends Temozolomide was expected to reach LD50 at 483.12 µg/ml.

Table 2-22 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (25.92)
Carmustine	Resistant
Temozolomide	Resistant (483.12)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

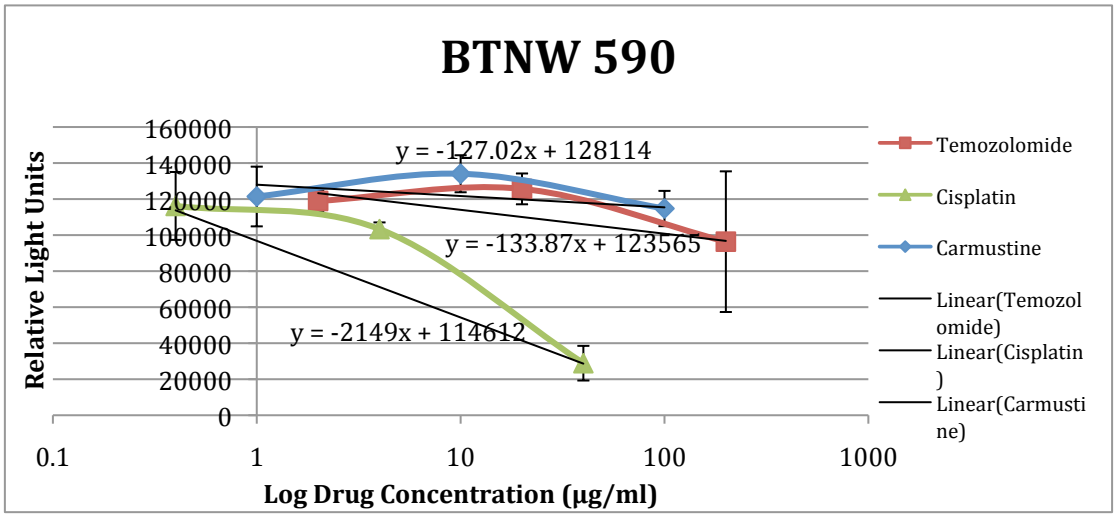

Figure 2-17 Dose response curves for BTNW 590 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

BTNW 597

Clinical History: This 73-year-old male presented with mild headache and unsteadiness. On examination he had a GCS of 15/15 with no neurological deficits. He received adjuvant radiotherapy (54 Gys) with concomitant Temozolomide followed by adjuvant Temozolomide chemotherapy treatment. He died after 108 weeks.

Radiology: CT revealed a right frontal intrinsic lesion.

Surgery: Craniotomy and debulking performed on the 21st of July 2010.

Histology: Histopathology revealed a Glioblastoma multiforme WHO grade IV.

Primary culture: Culture became confluent after 51 days.

Chemosensitivity results: ATP luminescent assay revealed resistance to Carmustine and Temozolomide. Cisplatin reached a 50% cell kill. (Table 2-23 and Figure 2-18). Given the observed dose response trends Both Carmustine and Temozolomide were expected to reach LD50 at 167 µg/ml and 628.82 µg/ml respectively.

Table 2-23 Chemosensitivity results for the tested drugs.

Drug	ATP assay LD50 (µg/ml)
Cisplatin	Sensitive (18.16)
Carmustine	Resistant (167)
Temozolomide	Resistant (628.82)

Resistance indicates that the calculated LD50 does not fall within the range of the tested drug concentrations. Calculated LD50 is shown in parenthesis.

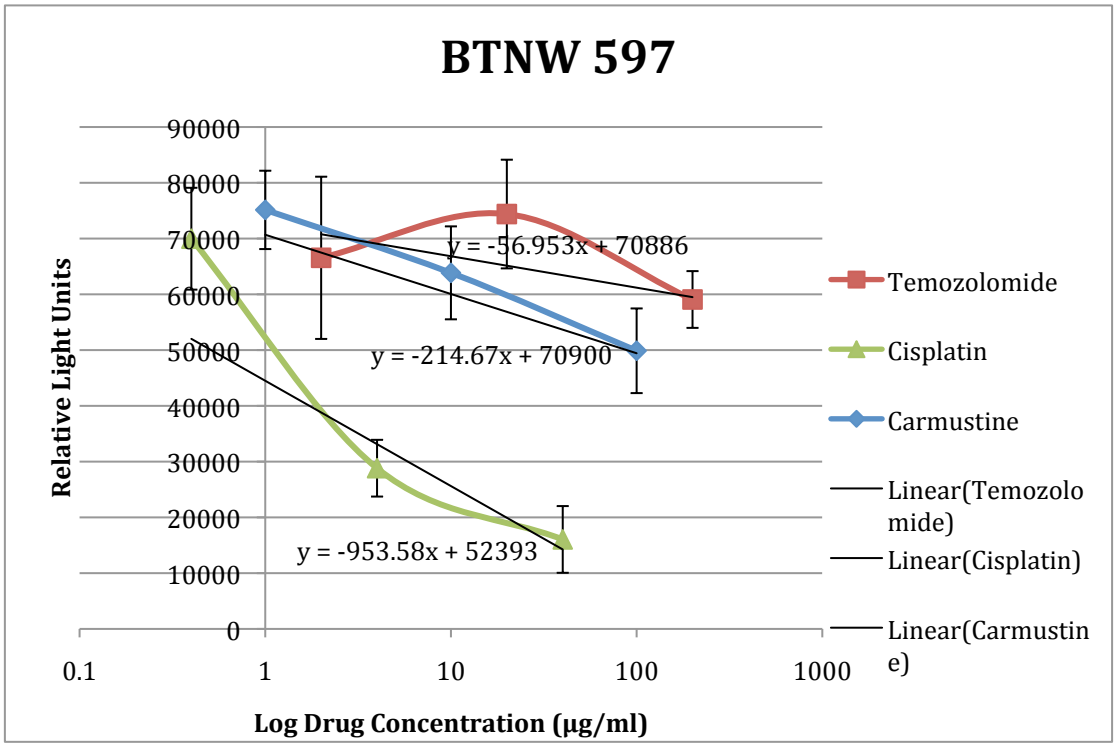

Figure 2-18 Dose response curves for BTNW 597 using the ATP assay. The data points are means of 4 repeats. The error bars represent standard deviations. Linear trend line was fitted on the data points to assist the calculation of LD50.

2.4.4 Summary of LD50 concentrations

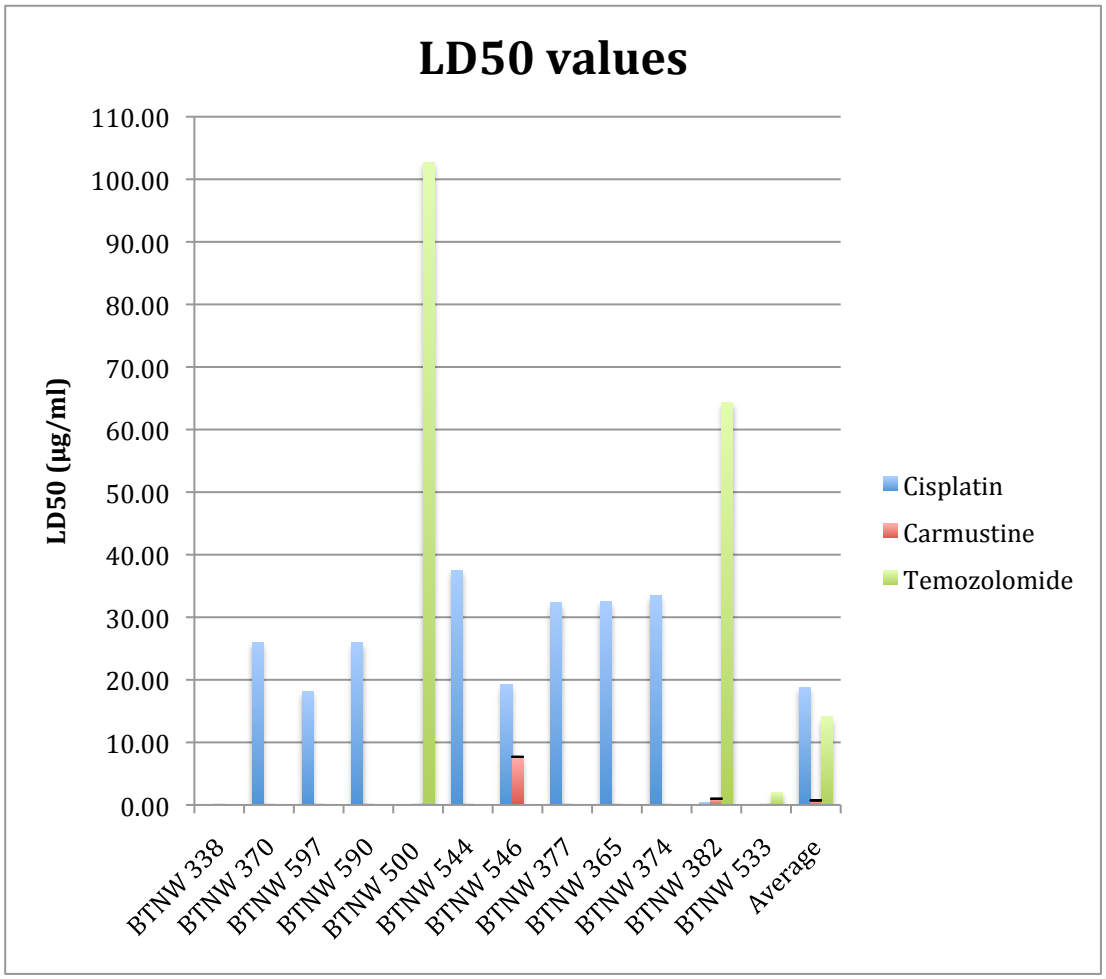

Figure 2-19 LD50 values for all patients. Values below the maximum drug concentration tested represent LD50s. Maximum tested drug concentrations were: Cisplatin 40 µg/ml, Carmustine 100 µg/ml, and Temozolomide 200 µg/ml.

Figure 2-19 summarises the calculated LD50 of each drug for all patients as well as the average LD50 with standard deviations. When the LD50 reaches the highest concentration used without producing 50% kill, the culture is defined as being chemoresistant. It is logical to think that the lower the concentration used as LD50, the more effective the drug was. The maximum concentration used for Cisplatin was 40 µg/ml, for Carmustine was 100 µg/ml and for Temozolomide was 200 µg/ml.

It is apparent from Figure 2-19 that almost all primary cultures but three were resistant to Temozolomide (BTNW 500, BTNW 382 and BTNW 533). Only one (BTNW 382) out of the three cultures sensitive to Temozolomide was also sensitive to Cisplatin and Carmustine, making this culture sensitive to all three drugs tested. The other two Temozolomide sensitive cultures (BTNW 500 and BTNW 533) were not sensitive to any other drug used.

Carmustine was the drug with least kill effect. In fact only BTNW 546 and BTNW 382 demonstrated sensitivity to it. Both these cultures responded also to Cisplatin.

Cisplatin was the drug with most kill effect as nine out of the twelve cultures were sensitive to it (BTNW 370, BTNW 597, BTNW 590, BTNW 544, BTNW 546, BTNW 377, BTNW 365, BTNW 374, BTNW 382). Only two of these nine cultures (BTNW 546 and BTNW 382) responded to other drugs in addition to Cisplatin. The remaining three cultures (BTNW 338, BTNW 500, BTNW 533) were resistant to Cisplatin. Of these three BTNW 500 and BTNW 533 were sensitive to Temozolomide whereas BTNW 338 was the only culture resistant to all drugs.

BTNW 382 was the most sensitive culture, responding to all drugs tested. It is important to emphasize that the concentrations required to produce kill effect to this culture were very small for all three drugs used (Cisplatin<0.4 μg/ml, Carmustine <1 μg/ml, Temozolomide 64 μg/ml).

BTNW 546 was the second most sensitive culture responding to both Cisplatin and Carmustine, again at very low concentrations (Cisplatin 19.29 μg/ml and Carmustine 7.7 μg/ml).

Based on these results, the average LD50 for Temozolomide was 14.08 μg/ml, Carmustine 0.73 μg/ml, Cisplatin 18.80 μg/ml.

2.4.5 Comparison of *in vitro* and *in vivo* data

In order to determine whether the results of the chemosensitivity studies exhibit any similarities with the clinical outcome, the *in vitro* results were compared to the clinical data for each patient.

BTNW 338 suffered from a Glioblastoma. In view of his age (71 years) and his poor Karnofsky score (<70), he did not receive any adjuvant treatment. The overall survival (OS) of this patient was five weeks. The primary culture grown from this patient was resistant to all the drugs used. This finding suggests agreement between the clinical course of this patient and the *in vitro* results obtained.

BTNW 370 suffered from a butterfly Glioblastoma. In view of the extent of the tumour she received palliative radiotherapy. Her overall survival was six weeks. The *in vitro* results of the primary culture of this patient revealed resistance to all drugs except from Cisplatin. The LD50 for Cisplatin was 25.49 µg/ml, five times the therapeutic dose of this drug. This finding suggests a possible agreement between the clinical course of this patient and the *in vitro* results obtained.

BTNW 597 suffered from a Glioblastoma. Despite his age (73 years), as he had a good Karnofsky score he received radiotherapy with concomitant Temozolomide, followed by adjuvant Temozolomide chemotherapy. His overall survival was 108 weeks. The *in vitro* chemosensitivity results for this patient revealed resistance to Temozolomide and Carmustine and sensitivity to Cisplatin. The LD50 for Temozolomide was expected to reach significance at 628.82 µg/ml. His OS survival outperformed the expected survival for his age, even with the Stupp chemotherapy regime. This observation suggests a possible agreement between the *in vitro* results obtained for this patient and his clinical course.

BTNW 590 suffered from a Glioblastoma. Despite his poor Karnofsky score (70), he received the Stupp chemotherapy regime. His overall survival was 80 weeks, outperforming the expected survival reported in the literature. The *in vitro* chemosensitivity results for this patient revealed sensitivity to Cisplatin and resistance to Carmustine and Temozolomide. However, Temozolomide was expected to reach significance at LD50 483,12 μg/ml. This observation could suggest a possible agreement between the clinical outcome for this patient with the *in vitro* results of his primary culture.

BTNW 500 suffered from a Glioblastoma. In view of her age (72 years) and poor Karnofsky score (<70), she received palliative radiotherapy. Her overall survival was 17 weeks. The *in vitro* chemosensitivity to Temozolomide obtained from the primary culture for this patient was 102.70 μg/ml. There was no other sensitivity to the remaining drugs used. Keeping in mind that she received palliative radiotherapy, the slightly longer OS without receiving any chemotherapy treatment, would indicate a possible predictive value of the *in vitro* results of this patient.

BTNW 544 suffered from a Glioblastoma. In view of his age (68 years) and good Karnofsky score (> 70), he received radiotherapy with concomitant Temozolomide, followed by adjuvant Temozolomide chemotherapy. OS for this patient was 89 weeks. The primary culture of this patient was sensitive to Cisplatin at 37.54 μg/ml. This value is nine times higher than the therapeutic concentration of this drug. His overall survival exceeds the one expected from the reported literature even for patients receiving the Stupp regime of chemoradiotherapy. His *in vitro* findings of chemosensitivity do not seem to agree with his clinical course.

BTNW 546 suffered from a Glioblastoma. As her Karnofsky score was 60, despite her age (50 years) she did not receive any adjuvant treatment. Her overall survival was only 3 weeks. The *in vitro* sensitivity results for this patient revealed sensitivity to both Cisplatin and Carmustine but not to Temozolomide.

As this patient did not receive any adjuvant treatment, it is difficult to draw any conclusions between *in vitro* and the clinical data available.

BTNW 377 suffered from a Glioblastoma. She was 45 years old at diagnosis with a good Karnofsky score (100). Intraoperatively she received six Gliadel wafers. This was then followed by adjuvant radiotherapy with concomitant Temozolomide and adjuvant Temozolomide chemotherapy. Her overall survival was 53 weeks. The primary culture obtained from this patient was only sensitive to Cisplatin (LD50 32.30 μg/ml). This concentration is eight times the therapeutic concentration of Cisplatin. The patient was treated with a combination of Carmustine wafers, radiotherapy and Temozolomide chemotherapy. Her OS was less than the expected OS of patients receiving similar treatment, giving the *in vitro* results a possible predictive value.

BTNW 365 suffered from a Grade II Fibrillary Astrocytoma with atypical features. She was 42 years old at diagnosis. Her Karnofsky score was >70. In view of the radiological appearances, this patient was treated as a grade III astrocytoma and received postoperative radiotherapy. She died after 163 weeks. The primary culture obtained from this patient showed sensitivity to Cisplatin (LD50 32.57 μg/ml) but not to the other drugs used. As this patient did not have chemotherapy treatment, it is difficult to draw any conclusions between the *in vitro* and the clinical data available.

BTNW 374 suffered from a Glioblastoma. In view of her young age (34 years) and good Karnofsky score (>70), she received radiotherapy with concomitant Temozolomide, followed by adjuvant Temozolomide chemotherapy. Overall survival for this patient was 30 weeks. The primary culture obtained from this patient was sensitive to Cisplatin with LD50 (33.50 μg/ml) eight times the therapeutic concentration for this drug. She received the Stupp chemotherapy regime. Her overall survival was worse than the one expected from the published literature. The *in vitro* results for this patient would concord with the observed clinical course.

BTNW 533 suffered from a Glioblastoma. In view of his age (76 years) and good Karnofsky score (>70), he received radiotherapy with concomitant Temozolomide, followed by adjuvant Temozolomide chemotherapy His overall survival was 21 weeks. The primary culture obtained form this patient was sensitive to Temozolomide (LD50 2 µg/ml). He received radiotherapy with the Stupp chemotherapy regime and unlike his *in vitro* response his OS was only 21 weeks.

BTNW 382 suffered from a Glioblastoma. In view of her age (75 years) and poor Karnofsky score (<70), she only received adjuvant radiotherapy. Her overall survival was 109 weeks. The primary culture obtained from this patient was sensitive to all drugs tested. The LD50 for the drugs used was low (Cisplatin<0.4 µg/ml, Carmustine <1 µg/ml, Temozolomide 64 µg/ml) potentially making her a suitable candidate for chemotherapy. Her clinical course would confirm the good predictive value of the results obtained from the *in vitro* chemosensitivity testing for her.

Overall the *in-vitro* results of this study are in agreement with the clinical outcome.

2.5 Discussion

Fifty-two tumour specimens were used in the present study in order to produce primary cell cultures. Twelve of those gave rise to cell cultures, which were tested with three chemotherapeutic agents. These drugs were Temozolomide, Carmustine and Cisplatin in three different concentrations (therapeutic concentration, 10-fold higher and 10-fold lower). The results have shown that Cisplatin and Temozolomide were effective at the highest concentration tested. Younger age was more responsive to these drugs. In addition, females were significantly more receptive to chemotherapy treatment. The LD50 concentration was calculated for each culture and the results were compared to the clinical data available for the donor patient.

A culture is defined arbitrarily as chemosensitive to a particular drug, when the concentration of the drug provokes at least 50% cell death when compared to the untreated population. However, the value that defines chemosensitivity is culture specific and therefore potentially highly variable between cultures.

The drugs tested in the present study are commonly administered as chemotherapeutic agents to patients treated in our Institution. Of these three drugs, the doses of Cisplatin and Carmustine were comparable to their peak plasma concentration, whilst the dose used for Temozolomide was equivalent to its Cerebrospinal Fluid (CSF) levels. The peak plasma concentration of each drug was defined as its therapeutic dose. In order to observe a dose response effect for each of these drugs, two additional concentrations, ten times lower and ten times higher, were included in this project.

The central nervous system (CNS) is a pharmacological sanctuary and as such peak plasma concentrations of drugs are not reflected in their CSF levels. For the purpose of this study, it was assumed that the levels of each drug in the Cerebrospinal Fluid (CSF) were similar to their peak plasma concentrations.

As there were no previously reported cisplatin peak plasma concentrations in patients with malignant gliomas it is difficult to draw any comparative conclusions. The closest study that could be found was by Himmelstein and coworkers who studied Cisplatin therapeutic concentrations in patients with head and neck cancers. They found that for these patients the therapeutic concentrations of Cisplatin was 6 μg/ml [149]. Another relevant study performed by Belliveau and coworkers reported cisplatin peak plasma concentrations of 4 μg/ml in five patients [150]. Unlike Cisplatin, the peak plasma concentrations of Temozolomide and Carmustine have been previously determined in patients with malignant gliomas. The reported concentrations were 4 μg/ml for Temozolomide and 0.1 μg/ml for Carmustine [144].

In the case of malignant gliomas, there could be several factors responsible for poor chemosensitivity. For instance, one of them could be the poor drug delivery to the tumour site. This could be due to poor penetration of the drug through the BBB, altered blood tumour supply or even impeded drug diffusion within the tumour mass. Another possible factor for poor response to chemotherapy could be a suboptimal intracellular drug concentration. For example, the tumour cells might not be able to take up the drug or have increased efflux mechanisms [151, 152]. Furthermore, cancer cells could suffer physiological alterations, which could affect processes such as apoptosis and drug metabolism (pro-drug activation or drug break down). Lastly, as chemotherapeutic agents are known to cause DNA damage, cancer cells that are able to rapidly repair their DNA could manifest chemoresistance. Poor chemosensitivity could be also a result of the failure of mechanisms that promote and execute apoptosis. For example, the tumour suppressor gene *p53* senses DNA damage by detecting DNA strand breaks caused by the drugs. Tumours with functioning *p53*, will promote cell death, whereas *p53* defective tumours could be more resistant to DNA damage [22].

As it is impossible to perform chemosensitivity testing in patients directly, this study uses primary glioma cultures that derived from tissue donated from those patients. The results indicate that Cisplatin 40 μg/ml together with

Temozolomide 200 µg/ml were found to be effective. This finding that Cisplatin is the drug with good kill effect is well documented in the literature both in malignant glioma primary cultures and cell lines [153-157]. Temozolomide on the other hand has been described as having variable effect. In those cases where Temozolomide was effective, the cell kill produced was found to be dose dependent [158, 159]. Both drugs demonstrated kill effect when used at the highest concentrations, which is in accordance with the reported literature [159]. Lower drug concentrations used were not found to be effective possibly due to the fact that not enough active compound was taken up by the tumour cells.

Temozolomide is now the standard of care for the treatment of malignant gliomas [160]. Standard of care is defined as the therapy that experts agree is appropriate, accepted and widely used. As such, Temozolomide has replaced the previous PCV (Procarbazine/CCNU a.k.a. Lomustine/Vincristine) chemotherapy regime for malignant gliomas. The dose as well as the frequency of administering Temozolomide chemotherapy is widely investigated and new methods have been proposed [161, 162]. PCV is currently used as a second line treatment after recurrence when previously treated with Temozolomide.

Cisplatin can also be used as second line treatment after recurrence. Despite this, it is mainly used in patients who develop further recurrence as third line treatment, when both Temozolomide and PCV have been used, and even repeated. The use of Cisplatin is limited by the considerable side effects that it produces, such as nephrotoxicity, ototoxicity, nausea, vomiting, electrolyte disturbance in the form of hypomagnesaemia, hypocalcaemia and hypokalaemia, haemolytic anaemia and myelotoxicity [163].

There is a clear need for predicting which is the most suitable drug dose combination for the individual patient affected by malignant glioma. For example, it is known that the response to Temozolomide depends on the methylation status of the MGMT (O6-methylguanine methyltransferase) promoter [98]. Although MGMT testing is available, it is not widely used in

clinical practice. Temozolomide is used for treatment in all patients affected by malignant gliomas regardless of their MGMT methylation status.

This study, although small (12 patients), demonstrates a good agreement between chemosensitivity testing and clinical outcome. Further studies are required in order to validate its predictive value in clinical practice. *In vitro* chemosensitivity studies, if successful, could provide an additional line of evidence in supporting the choice of chemotherapy drug and concentration tailored to each patient.

The mode of action of all three drugs tested is by causing DNA damage (see Chapter 1). Facilitating the access to these drugs to the DNA, could improve their efficacy. Ways to achieve this would be by enhancing the drug formulation, improving drug delivery and rendering the target cells more susceptible to the drug. Slow release formulations and longer half-life could lead to improved stability of the drug's active component. Similarly, disruption of the intercellular tumour matrix and facilitating both transmembrane and intracellular drug transport would improve the drug's access to its substrate. Furthermore, inhibition of the metabolic pathways that cause drug deactivation and suppression of DNA repair mechanisms would render cells more vulnerable to the drug's activity. There are several studies already focusing on the enhancement of the overall effect of chemotherapy drugs [158, 164-167].

As expected, this study has found considerable variability in the response of individual patients to the tested drugs and concentrations. For example, Cisplatin was effective in nine patients, but three others were resistant. Even between the nine sensitive patients, there was great variation of their LD50 concentration. The variability observed could be a result of the extensive genetic heterogeneity of the malignant glial cells. For instance, malignant glioma cells were found to have several genetic alterations including 1p/19q loss of heterozygosity, EGFR amplification and isocitrate dehydrogenase 1 (IDH1) mutations [168].

Another possible cause for the variability observed could be related to the tissue specimen cultures. For example, the specimen arriving in the laboratory could be part of the necrotic rather than the actively dividing area of the tumour, and as such would not give rise to a culture that is representative of the tumour. Furthermore, as there are quiescent cells in the culture, the chemosensitivity measurements might not be entirely accurate. Quiescent cells will not be killed by the drug, but still be accounted for by the ATP assay measurement.

Assay methodology could also be accounted for the variability observed. Parameters like drug exposure time, dosing frequency, initial number of cells and number of replicates could potentially affect the observed variability. Standard laboratory procedures such as tissue culturing, sterile handling as well as good laboratory practices would reduce the likelihood of variability between samples.

In the present study, chemosensitivity to the drugs tested was evaluated using the LD50 concentration. The average LD50 for Temozolomide was 164.08 μg/ml (± 68.49), Carmustine 84.06 μg/ml (±37.26) and Cisplatin 28.80 μg/ml (±11.79). Studies on mice and rats have reported LD50 of 1937 mg/m^2 after oral administration of Temozolomide. When Temozolomide was administered via an intraperitoneal injection, the LD50 was 1414 mg/m^2 [169]. A study performed in our Institution using short-term primary cultures of malignant gliomas, reported LD50 concentrations for Cisplatin and Carmustine as being 1-37 μg/ml and 10-90 μg/ml respectively [168]. Given the great variability found in the present study of the LD50 between patients, a mean LD50 value would not be very informative. On the contrary, the LD50 value for each individual would be more appropriate and relevant to the patient's chemosensitivity profile. Nevertheless, calculating the LD50 for each drug was not always straightforward. A larger number of tested concentrations as well as more replicates would facilitate a more precise calculation of the LD50. The only constraint to this approach would be the limited number of available cells from the primary culture.

The novelty of this study lies in the use of primary glioma cultures at passage 0 as opposed to short-term glioma cultures or glioma cell lines. This type of culture is more suitable for chemosensitivity studies as it is more representative of the considerable genetic heterogeneity of malignant gliomas between patients. On the contrary, glioma cell lines carry a more homogenous genetic load, as with every passage there is clonal selection of rapidly dividing cells. Such genetic misrepresentation in cell lines poses a limit as to their usefulness in research and clinical studies.

Age was another factor that influenced chemosensitivity in the present study. Overall the under 65 years age group responded better to chemotherapy treatment with the aforementioned drugs although it was hard to draw solid conclusions based on these findings. These results concord with the reported literature where there is no consensus on the role of age in relation to *in vitro* chemosensitivity [126, 170, 171]. On the other hand, age is a known prognostic factor in gliomas in clinical practice. Several studies have identified that amongst others, age is an important factor that influences survival in patients with high-grade glioma [172-174]. This could be due to the accumulation of more genetic defects over a period of time or because of the reduced capacity of aged cells to repair DNA damage.

The results of this study have also shown that females were more sensitive to the tested drugs at the different doses than the males. Similar findings have been reported in the literature, but the cause of this observation is still unclear [175].

Another advantage of the present work is the availability of clinical data that enabled a retrospective evaluation of the chemosensitivity results. There was good agreement between *in vivo* and *in vitro* results for nine out of the twelve patients tested. For example, BTNW 377 received Temozolomide chemotherapy but survived only 53 weeks, despite her young age. *In vitro* chemosensitivity testing for the same patient revealed that she was resistant to Temozolomide

but sensitive to Cisplatin (LD50 32.30 µg/ml). The results of the chemosensitivity testing for this patient were predictive of her bad clinical outcome when she was treated with what is currently best practice. One could speculate that her outcome might have been different if she was offered Cisplatin chemotherapy instead.

An important factor that guides chemotherapy treatment is the side effects of drugs used. Patients are closely monitored during the course of their treatment in an attempt to predict the onset of side effects not clinically apparent. Chemosensitivity testing would avoid the use of drugs, which would not be beneficial to the patient as well as their side effects. In order for *in vitro* chemosensitivity results to translate into clinical practice, the concentrations tested in the present study were within the range of those used clinically. Such methodology makes this study relevant to clinical practice. Another potential benefit of *in vitro* chemosensitivity testing is its predictive value when it comes to the choice of chemotherapy treatment. For example, chemosensitivity testing has shown that BTNW 382 was very sensitive to all three drugs. However, she did not receive any chemotherapy treatment because of her age (75 years) and Karnofsky score (60). Should the chemosensitivity testing results have been taken into account, this patient might have had a better clinical course.

Growing primary glioma cultures and subsequent chemosensitivity testing is a challenging technique and requires specialised skills. Such expertise would establish standard operating procedures as well as tackle issues such as tissue availability, stagnant or slow growth and contamination control more effectively. For that matter, brain tumour banks are invaluable assets as they provide access to the much-needed tissue.

Overall this study suggests that chemosensitivity testing can be a valuable tool to complement clinical practice. However, more studies are required to strengthen the findings of the present work, so that chemosensitivity testing becomes routinely used in chemotherapy treatment.

3 TEMOZOLOMIDE EVALUATION IN GLIOMA CELL LINES

3.1 Introduction

Glioblastoma is the most malignant tumor of central nervous system neoplasms with poor overall survival. Treatment of glioblastomas is challenging and very little progress was made until the last decade. A phase III randomised trial conducted by the European Organisation for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada Clinical Trials Unit (NCIC), revolutionised chemotherapy treatment of newly diagnosed glioblastoma. The results of this trial, published in 2005, showed a significant benefit of both median and two-year survival of patients treated by radiotherapy with concomitant chemotherapy with Temozolomide (TMZ), followed by adjuvant Temozolomide chemotherapy [96]. The survival benefit continued through to 5 years of follow-up, with a few patients surviving longer than 5 years [99]. The "Stupp regime", named after Roger Stupp who conducted the trial, includes concomitant daily Temozolomide during initial radiotherapy treatment, followed by six cycles of adjuvant Temozolomide (for five days during a 28-day cycle). In another study, Temozolomide was also found to improve survival in patients with recurrent glioblastoma [176].

Temozolomide is an Imidazotetrazinone methylating agent, which is 100% bioavailable after oral administration and is excreted by the kidney. It has very few side effects making it well tolerated by patients. Temozolomide hydrolyses spontaneously to its active metabolite 5-(3-methyl triazen-1-yl) imidazole-4-carboxamide (MTIC) at physiological pH. MTIC is degraded within seconds into methyldiazonium and into an inactive metabolite 5-aminoimidazole-4-carboxamide (AIC).

MTIC acts by methylating O6 and alkylating N7 position of guanine. When DNA mismatch repair enzymes attempt to excise O6-methyl-N7-alkylguanine, they generate single- and double-strand DNA breaks. These lead to activation of

apoptotic pathways, which cause cell death. Agarwala and coworkers have reported that the double strand DNA breaks are formed in a cell when Temozolomide is present for at least two cell cycles [177].

Prior to Temozolomide there had been no significant improvement of survival of glioma sufferers for almost twenty years. Temozolomide has been a major breakthrough for treating glioma patients, offering a survival benefit without the unwanted side effects of previous chemotherapeutic agents used.

Despite its efficacy in clinical practice, Temozolomide does not appear to be as effective in *in vitro* chemosensitivity studies. This observation together with the anecdotal reports of the poor *in vitro* effect of Temozolomide, initiated a series of experiments that investigated the reason behind this observation.

3.2 Hypothesis

There were two hypothesis formed that could explain the poor *in vitro* effect of Temozolomide. The first one proposed that as Temozolomide is inactivated too rapidly it does not have enough time to produce an effect on the cells. The second hypothesis proposed that the cells require a longer exposure to Temozolomide in order to have an observable kill effect.

3.3 Aims of the following study

This chapter investigates the poor *in vitro* effect of Temozolomide on primary cell cultures, which was observed in the previous chapter (Chapter 2 Chemosensitivity Testing in Primary Glioma Cultures).

Based on the fact that Temozolomide has a short half-life, this study investigates as to whether daily application of Temozolomide, as opposed to a single dose, would improve its kill efficiency.

In addition, given that Temozolomide causes DNA damage, this chapter also examines the possibility of improving its efficacy in *in vitro* studies by cell cycle synchronisation prior to Temozolomide application.

Furthermore, it aims to optimise Temozolomide's *in vitro* use.

3.4 Materials and methods

Six established glioma cell lines were used to determine the effect of Temozolomide *in vitro*. These were the U373, U257, IN1265, IN859, IN077, and 1321N1. The cell lines were synchronised into the "S" phase of their cell cycle by repeated rounds of feeding and starvation. The synchronisation of the cell lines was confirmed by DNA analysis using a Flow Cytometer (Becton Dickinson (BD) FACSAria) (Figure 3-1). Once the cell lines were synchronised, they were treated with either a single or a daily dose of Temozolomide. Daily cell counts were performed for five days and compared to those of the untreated population. Cisplatin was used as a positive control and Carmustine (BCNU) was used as a negative control.

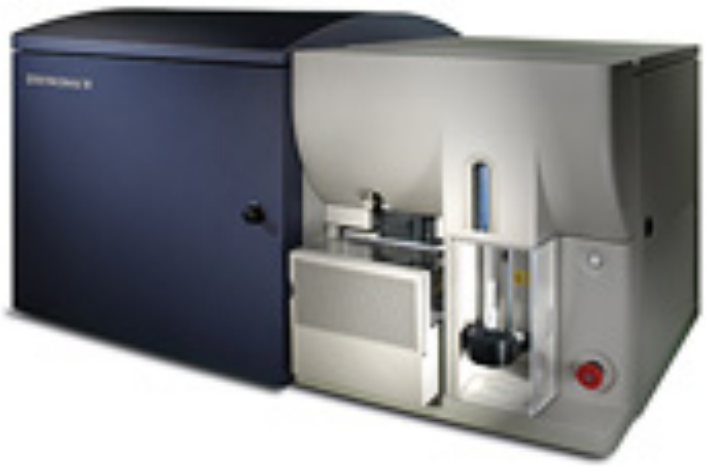

Figure 3-1 BD FACSAria III Adapted by [178].

3.4.1 Preparation of cells from flow cytometry to demonstrate cell cycle synchronization

Cells from each cell line were counted to 500.000 cells per flask and the appropriate volume was then placed in three 25 cm^3 flasks. All three flasks were returned to the incubator and the cells were allowed to attach and grow for 48 hours. Flask No 1 was trypsinised as per protocol and the sample was then taken for Propidium Iodide staining. The complete growth medium was aspirated from flasks No 2 and No 3 and replaced with equal volume of serum-free media. Both flasks were then returned to the incubator for 24 hours. After 24 hours flask No 2 was trypsinised and taken for Propidium Iodide staining. The serum free medium was aspirated from flask No 3 and replaced with the same volume of complete growth medium. Flask No 3 was then placed in the incubator for another 24 hours. Following the 24 hours, flask No 3 was trypsinised and stained with Propidium Iodide as per protocol.

3.4.2 Preparation of cultures to test delayed response of cell lines to Temozolomide

Twelve 35mm Petri dishes were labelled as C1, 1, 1S, C2, 2, 2S, C3, 3, 3S, C4, 4, 4S and two as medium. A cell suspension of each cell line was prepared containing 2000 cells/ml and 1ml of the cell solution and 1ml of complete growth medium were added to each petri dish. All petri dishes were returned to the incubator for 24 hours to allow cells to attach. After 24 hours, the complete growth medium was aspirated from all the dishes. Two millilitres of new complete growth medium were added to dishes C1, 1, C2, 2, C3, 3, C4, 4 and medium. Two millilitres of serum-free medium were added to dishes 1S, 2S, 3S and 4S. All dishes were returned to the incubator for a further 24 hours. The media from all of the dishes was then aspirated and replaced with 2ml of complete growth media. All dishes were returned to the incubator for 24 hours. The complete growth media from all of the dishes was aspirated except from 'Media' and replaced with 1.2ml (1200µl) of new media. Then 0.8 ml (800 µl) of 10 µg/ml Carmustine solution was added to dishes C1, C2, C3 & C4 and 0.8 ml

(800 µl) of 20 µg/ml Temozolomide solution were added to all other dishes. The culture dishes were returned to the incubator for 24 hours. After 24 hours, dishes C1, 1 and 1S were washed with Hanks to remove dead cells. They were subsequently trypsinised together with one of the 'Media' dishes and the cells were counted in the coulter counter as per protocol. This step was repeated every 24 hours with the next number of dishes. Finally, the last 'Medium' dish was trypsinised at the same time as C4, 4 and 4S and the cells were counted.

3.4.3 Propidium Iodide nucleic acid stain

Phosphate Buffered Saline (0.01M), pH 7.2

This solution contained 0.74 g di-Sodium Phosphate anhydrous, 0.22 g Potassium di-Hydrogen Orthophosphate, 3.6 g Sodium Chloride and 500 ml Distilled Water.

Propidium Iodide Staining Solution

This solution contained 350 µl PBS, 50 µl of 500 µg/ml Propidium Iodide and 5 µl of 10 mg/ml RNase. *(Ribonuclease A from bovine pancreas, Cat No R6513 10 mg. This was dissolved in sterile water to make up 10 mg/ml concentration.)*

Method

The cells were trypsinised for 5 minutes, and then two volumes of the complete growth medium were added to inactivate the trypsine. Then the trypsinised culture was centrifuged for 5 minutes at 250g, the supernatant was pipetted off and the cells were resuspended in 1ml PBS. The cells were then centrifuged again, the PBS was pipetted off and replaced with 1 ml of ice-cold 70% ethanol drop wise whilst vortexing to prevent clumping. This cell suspension could be

safely stored at –20°C until staining and analysis. If proceeding with the staining, the cells were centrifuged for 5 minutes at 1000 RPM, the 70% ethanol was pipetted off and the cells were resuspended in 1 ml ice-cold PBS. The cells were centrifuged again, the PBS was pipetted off and replaced with Propidium Iodide (PI) staining solution (final volume 500 µl / 500,000 cells). The cells were then incubated at 37°C for 30 minutes and then kept at 4°C in the dark until Flow Cytometry analysis, preferably on the same day. The Flow Cytometer used was an A Becton Dickinson (BD) FACSAria with a 488nm blue laser fitted with a doublet discrimination module. The emitted PI fluorescence was detected using the PE detector in conjunction with the 575/26 band pass filter. This detector collects a fluorescence wavelength of 575 with a 26nm range (from 562-588 nm). Ten thousand single cells were acquired per sample at the lowest flow rate possible. A Forward Scatter (FCS) and a Side Scatter (SSC) plot was used to measure relative size and internal complexity of the cells examined. Internal complexity is the presence of granules or lobed nuclei within the cells. Cells with high internal complexity, have a higher side scatter. This FCS/SSC plot was used to ensure the whole cell population was included in the analysis, excluding large clumps or debris. This was achieved by placing a region around the cell population to be analysed. A doublet discrimination plot (PE-W/PE-A) was also used, in order to ensure the analysis of single cells rather than cell aggregates. A DNA content histogram was obtained from the area marked. Boxes around the peaks of the histogram were inserted manually in the appropriate place. This way the percentage of cells in each stage of the cell cycle (G_0G_1, S and G_2M) was estimated. More accurate calculation of each stage of the cell cycle could have been made with the use of the Modfit software. At the time of these experiments this was not available in our laboratory.

3.5 Results

3.5.1 Flow cytometric analysis of cell cycle distribution

Flow cytometry was used to monitor the cell synchronisation process. Samples were exposed to a round of feeding, starvation followed by further feeding prior to their analysis on the flow Cytometer. The cell line U373 is used as an example to illustrate the three different stages of the synchronisation process as shown in the figure below.

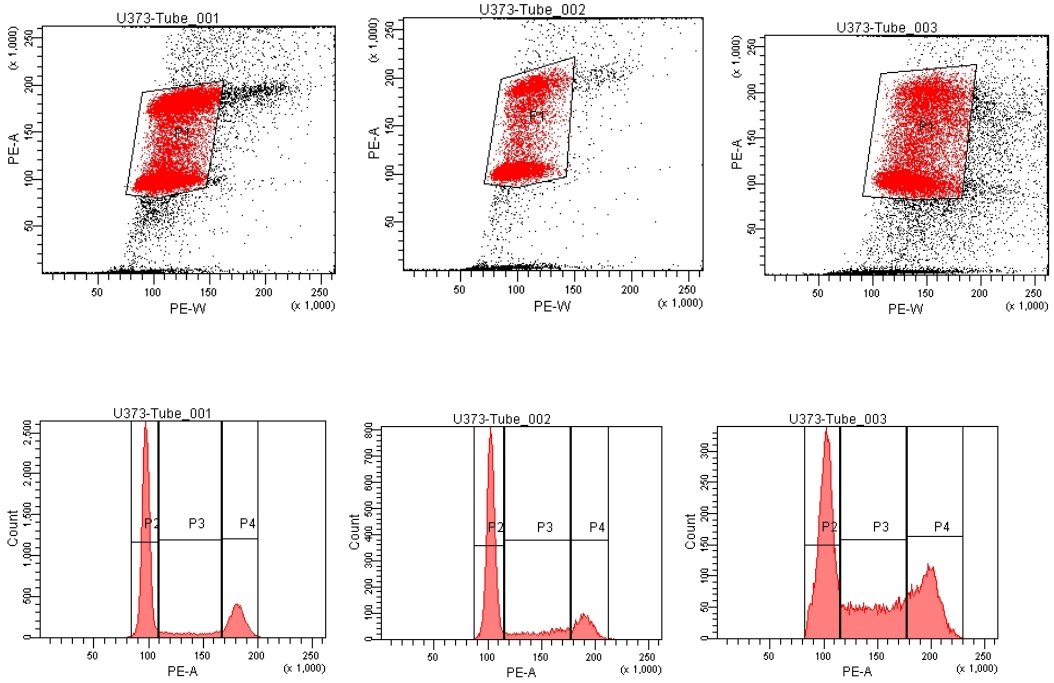

Figure 3.1 Flow cytometric analysis of the cell-cycle distribution of U373. Top row illustrates the doublet discrimination plot associated with the Cellquest software used to identify the single cell population denoted by region P1 (red area inside polygon). Cells examined were part of a culture after feeding (left panel), starvation (middle panel) and refeeding (right panel). Bottom row illustrates the DNA histogram obtained from region P1 analysed to calculate the relative percentage of the population in G_0G_1, S and G_2M. Regions P2, P3 and P4 correspond to G_0G_1, S and G_2M respectively.

Table 3-1 Percentage of cells in the different cell cycle phases during a round of feeding, starvation and refeeding for cell line U373.

Population (cell cycle phase)	Feeding (%)	Starvation (%)	Refeeding (%)
P1 (all)	83.5	77.3	56.4
P2 (G_0G_1)	69.6	69.2	45.6
P3 (S)	8.8	14.2	26.0
P4 (G_2M)	21.4	16.0	27.4

As seen form the Table 3-1 above, after a round of feeding, starvation and refeeding, the number of cells in the S phase has increased from 8.8% to 26%. This is important as these cells are targeted during Temozolomide treatment. In addition, quiescent cells that were resting into G_0G_1 phase have dropped from 69.6% to 45.6% indicating that they are re-entering the cell cycle and thus becoming vulnerable to Temozolomide.

3.5.2 Temozolomide analysis

Temozolomide is used as first line chemotherapy treatment for patients with glioblastoma. However, in *in vitro* chemosensitivity studies on primary human glioblastoma cultures it does not have the same effectiveness. Six established cell lines were used to determine the effect of Temozolomide *in vitro*. The cell lines were treated either with repeated daily doses of Temozolomide or with a single treatment at the onset of the experiment. In addition, as Temozolomide is known to cause DNA breaks its efficacy was tested during the DNA replication phase (S-phase) where it was expected to be most effective. Like above, the drug was delivered to the synchronized cells repeatedly on a daily basis (T"s" daily), or only once at the onset of the experiment (T"s" once). As negative control each line was treated with BCNU (Carmustine). Cisplatin was also included in the experiment as positive control.

To compare the effect of the single treatment versus the "Daily" one, the Kolmogorov–Smirnov statistical test (KS test) was used. The Kolmogorov–Smirnov statistic quantifies the distance between the empirical distribution functions of two samples. In this case, the KS test was used for the pair-wise comparison of the cumulative distributions of: a) Temozolomide "once" *vs.* Temozolomide "daily", b) Temozolomide synchronised "daily" *vs.* Temozolomide "daily", c) Temozolomide synchronised "once" *vs.* Temozolomide "once" and d) Temozolomide synchronised "once" *vs.* Temozolomide synchronised "daily".

In terms of evaluating statistical significance given a calculated p-value, both significance levels $\alpha=0.001$ (conservative) and $\alpha=0.05$ (widely used) were considered. The null hypothesis is that the two samples have a similar cumulative distribution. If a test of significance gives a p-value lower than the significance level α, the null hypothesis is rejected and the two samples can be considered as being statistically significantly different.

Statistical package

The statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.15.1) running under Windows Vista OS with 2GB RAM [148]. All the statistical tests used here were included in the R Stats package.

3.5.3 Comparison of single *vs.* daily treatment

To compare the effect of the single treatment versus the "Daily" one, the KS test was used to analyse the data. The results of the comparison between the two dosing methods in the kill efficiency for each drug after 4 days of incubation is shown in Table 3-2 and Figure 3-2 and Figure 3-3.

Table 3-2 *Comparison of once versus daily dosing methods in the kill efficiency of Temozolomide.*

	ks distance	p-value
Comparing Plate T (Once *vs.* daily)	0.352	**<0.05**
Comparing Plate T"s" (Once *vs.* daily)	0.222	0.139

Temozolomide on non-synchronised cells (T) and Temozolomide on synchronised cells (T"s") after four days of incubation. p-values as determined by the KS test. Significant results are shown in bold.

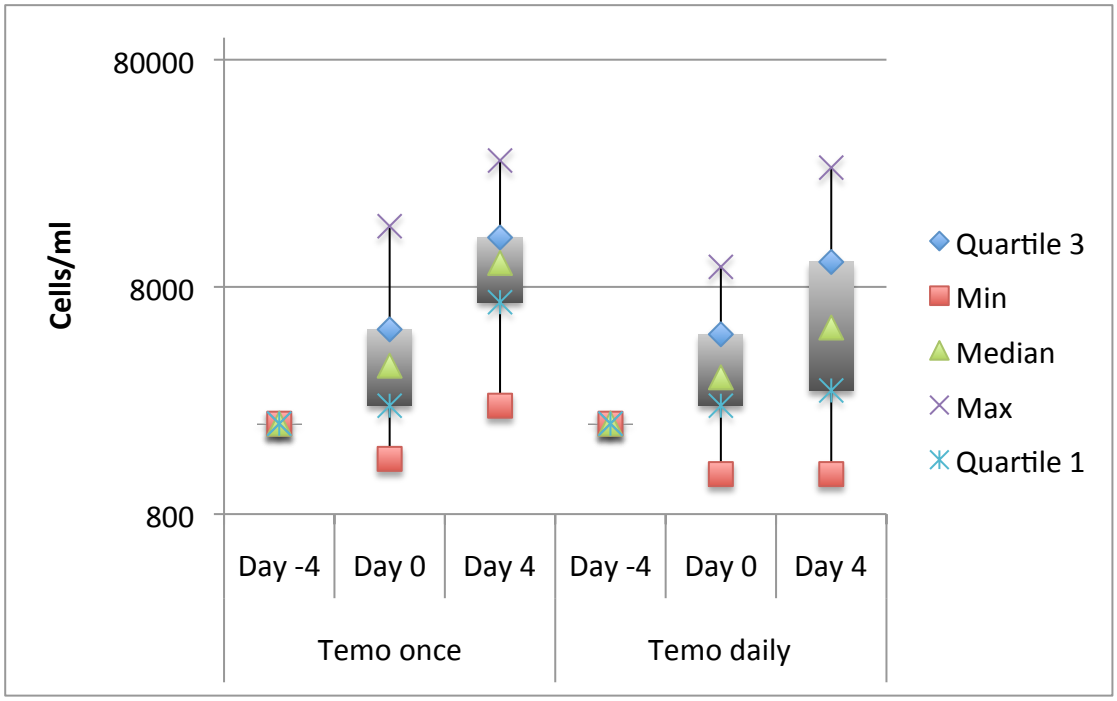

Figure 3-2 *Comparison of single vs. daily dose of Temozolomide on non-synchronised cells after four days of incubation. Temo once: single dose of Temozolomide; Temo daily: daily dose of Temozolomide.*

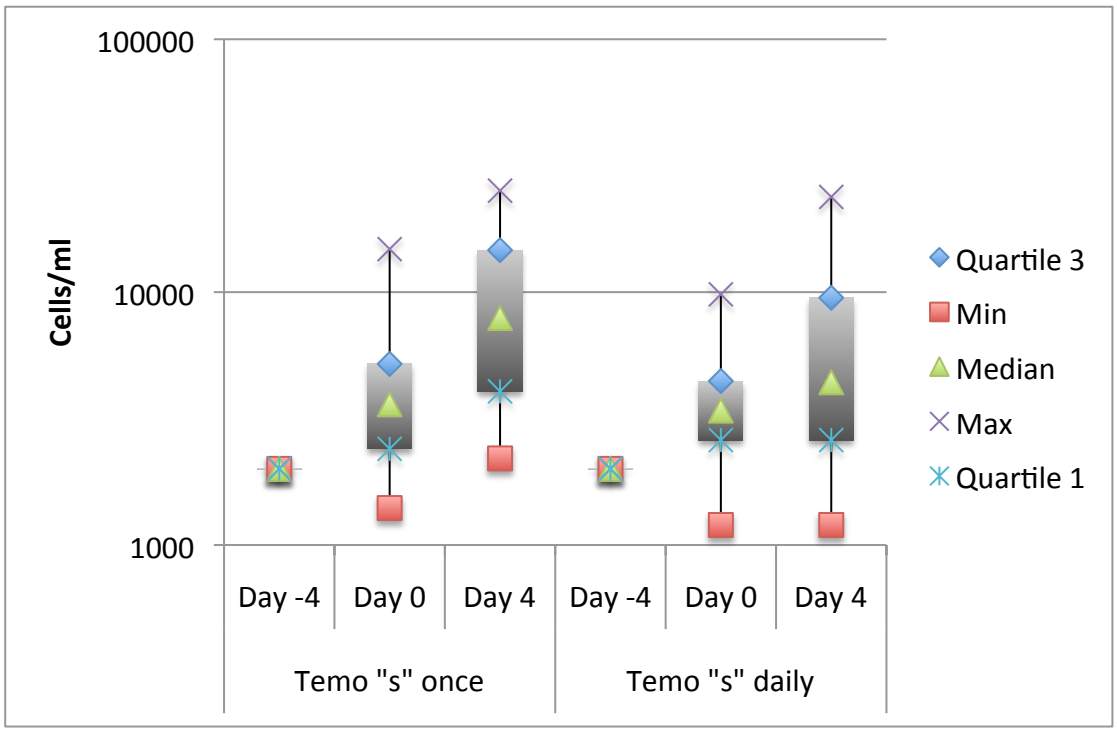

Figure 3-3 ***Comparison of single vs. daily dose of Temozolomide on synchronised cells after four days of incubation. Temo "s" once: single dose of Temozolomide on synchronised cells; Temo "s" daily: daily dose of Temozolomide on synchronised cells.***

As shown above, the dosing method of Temozolomide's application had a significant effect (α=0.05) suggesting that the length of drug exposure and the frequency of application did seem to affect the kill effect of the drug. This didn't appear to be the case for Temozolomide on a synchronized cell culture at α=0.05.

3.5.4 Effect of cell culture synchronization on Temozolomide's kill efficacy during single application.

To determine the effect of the cell synchronisation on the kill efficiency of a single dose of Temozolomide, we compared the two datasets using the KS test. The results from this comparison are summarised on Table 3-3 and Figure 3-4.

Table 3-3 *Comparison of synchronisation and single dosing in the kill efficiency of Temozolomide.*

Comparing Plate T once *vs.* T "s" once	ks distance	p-value
Day 1	0.185	0.313
Day 2	0.148	0.594
Day 3	0.167	0.441
Day 4	*0.241*	*0.088*

Comparison of Temozolomide single dose on synchronised versus non-synchronised culture cells from day 1 to day 4 of incubation (p-value as determined by the KS test).

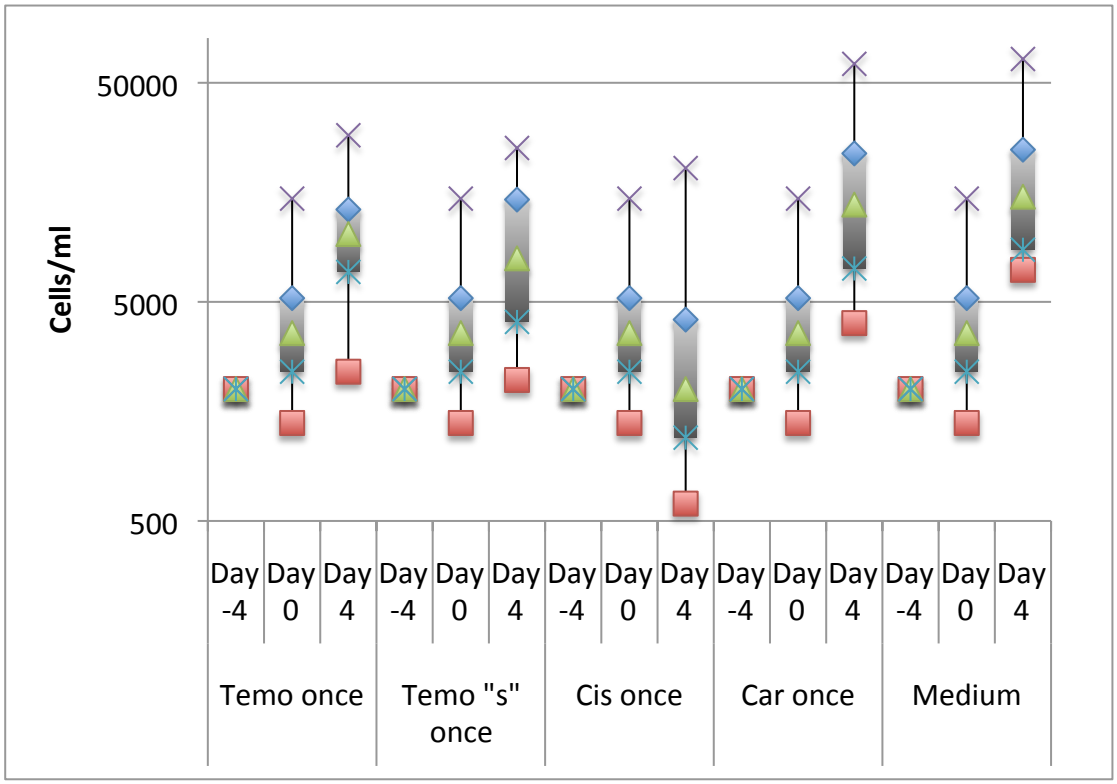

Figure 3-4 *Comparison of single dose of Temozolomide on synchronised vs. non-synchronised cells after four days of incubation. Temo "s" once: single dose of Temozolomide on synchronised cells; Temo once: single dose of Temozolomide on non-synchronised cells.*

Based on these results, there was no significant difference between the synchronous and asynchronous cell cultures when they were treated with a single dose of Temozolomide. Still, from day 2 of the incubation onwards, the ks-distance was gradually increasing and on day 4 the p-value was dropped close to the cut off value of α=0.05 (0.088). One could speculate that further incubation might have led to further reduction of the p-value but this remains to be tested in the lab.

3.5.5 Effect of cell culture synchronization on Temozolomide's kill efficacy during daily application.

Similarly to the single dose, the effect of the cell synchronisation when Temozolomide was applied on a daily basis on both synchronous and asynchronous cell cultures was tested. The two datasets were compared using the KS test and the results are summarised on Table 3-4 and Figure 3-5.

Table 3-4 *Comparison of synchronisation and daily dosing in the kill efficiency of Temozolomide.*

Comparing Plate T daily *vs.* T "s" daily	ks distance	p-value
Day 1	0.222	0.139
Day 2	0.130	0.755
Day 3	0.204	0.213
Day 4	0.111	0.893

Comparison of daily dosing of Temozolomide on synchronous and non-synchronised culture cells from day 1 to day 4 of incubation (p-value as determined by the KS test).

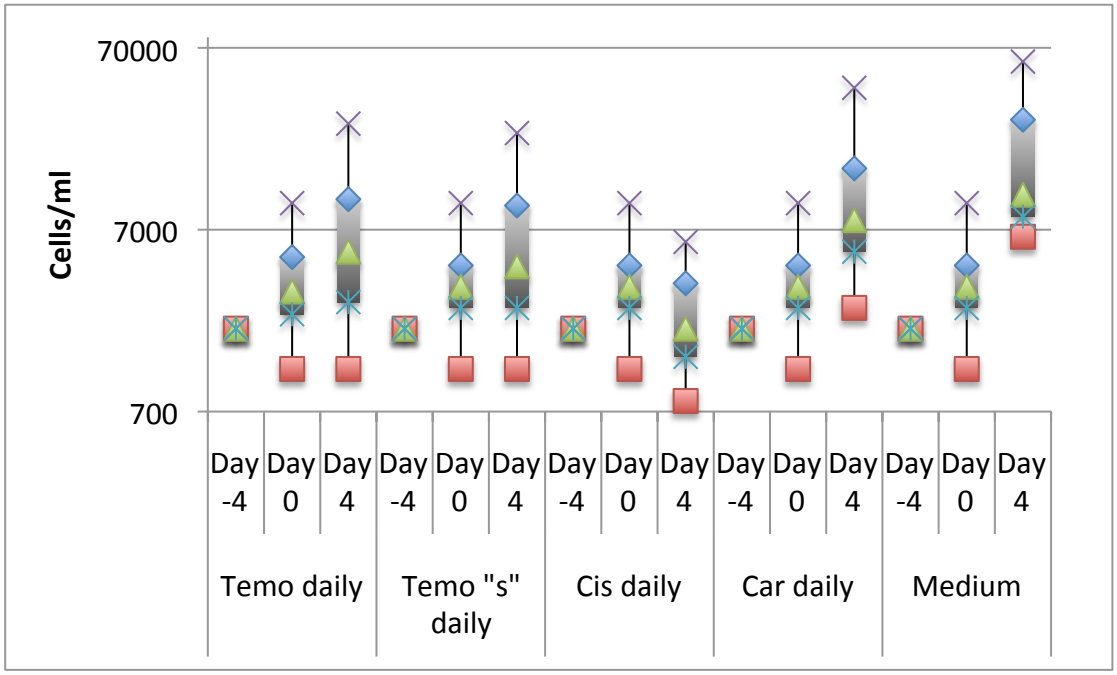

Figure 3-5 Comparison of daily dosing of Temozolomide on synchronised vs. non synchronised cells after four days of incubation. Temo daily: daily dose of Temozolomide on non-synchronised cells; Temo"s" daily: daily dose of Temozolomide on synchronised cells.

From these results we can conclude that the kill effect of the daily dosing of the drug didn't seem to be affected by the cell culture synchronisation (α=0.05).

The last two experiments suggest that given the experimental setup the cell culture synchronization does not have a significant impact on the Temozolomide's kill efficiency for the given concentration, frequency of application, and duration of the experiment as determined by the KS statistical test.

3.5.6 Comparison of cell culture synchronization and frequency of application on Temozolomide's kill efficacy (T "s" once vs. T daily).

In order to establish whether cell synchronisation and frequency of drug application were equally influential to Temozolomide's kill efficacy, the kill efficiency of a single dose of Temozolomide on synchronised culture cells was compared to that of a daily dose of Temozolomide on a non-synchronised cell population. If either the synchronisation or the frequency of application had a

stronger role than the other, the kill efficiency of Temozolomide between the two cell populations would be significantly different. The two datasets were compared using the KS test and the results are summarised on Table 3-5 and Figure 3-6.

Table 3-5 *Comparison of synchronisation and dosing in the kill efficiency of Temozolomide.*

Comparing Plate T "s" once *vs.* T daily.	ks distance	p-value
Day 1	0.222	0.139
Day 2	0.222	0.139
Day 3	0.259	0.053
Day 4	0.185	0.313

Comparison of Temozolomide single dose on synchronised versus Temozolomide daily dose on non-synchronised culture cells from day 1 to day 4 of incubation (p-value as determined by the KS test).

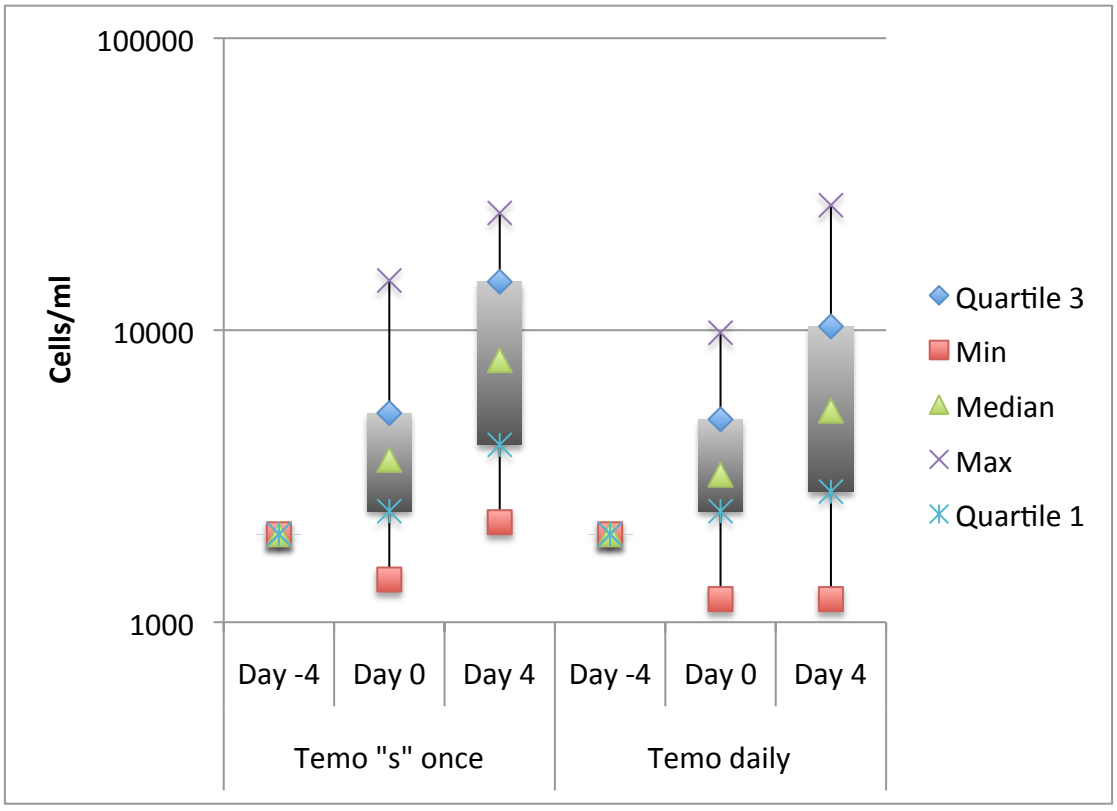

Figure 3-6 *Comparison of Temozolomide single dose on synchronised versus Temozolomide daily dose on non synchronised culture cells after four days of incubation. Temo daily: daily dose of Temozolomide on non-synchronised cells; Temo "s" once: single dose of Temozolomide on synchronised cells.*

Based on these results there was no significant difference between the effect caused by cell synchronisation and frequency of drug application to Temozolomide's kill efficacy when comparing single application on synchronised cells to daily application on non-synchronised cells. Still, on day 3 of the incubation, the p-value dropped close to the cut off value of $\alpha=0.05$ (0.053) before it increased again on day 4.

3.5.7 Comparison of cell culture synchronization and frequency of application on Temozolomide's kill efficacy (T once vs. T "s" daily).

In order to confirm that synchronisation and daily application had an additive effect, the kill efficiency of a single dose of Temozolomide on non-synchronised culture cells was compared to that of a daily dose of Temozolomide on a synchronised cell population. If synchronisation and frequency of application had no effect, the kill efficiency of Temozolomide between the two cell populations would be similar.

If either the synchronisation or the frequency of application had a stronger role than the other, the kill efficiency of Temozolomide between the two cell populations would be significantly different. The two datasets were compared using the KS test and the results are summarised in Table 3-6 and Figure 3-7.

Table 3-6 ***Comparison of synchronisation and dosing in the kill efficiency of Temozolomide.***

Comparing Plate T once *vs.* T "s" daily.	ks distance	p-value
Day 1	0.241	0.087
Day 2	0.296	**<0.05**
Day 3	0.352	**<0.05**
Day 4	0.352	**<0.05**

Comparison of Temozolomide single dose on non-synchronised versus Temozolomide daily dose on synchronised culture cells from day 1 to day 4 of incubation (p-value as determined by the KS test).

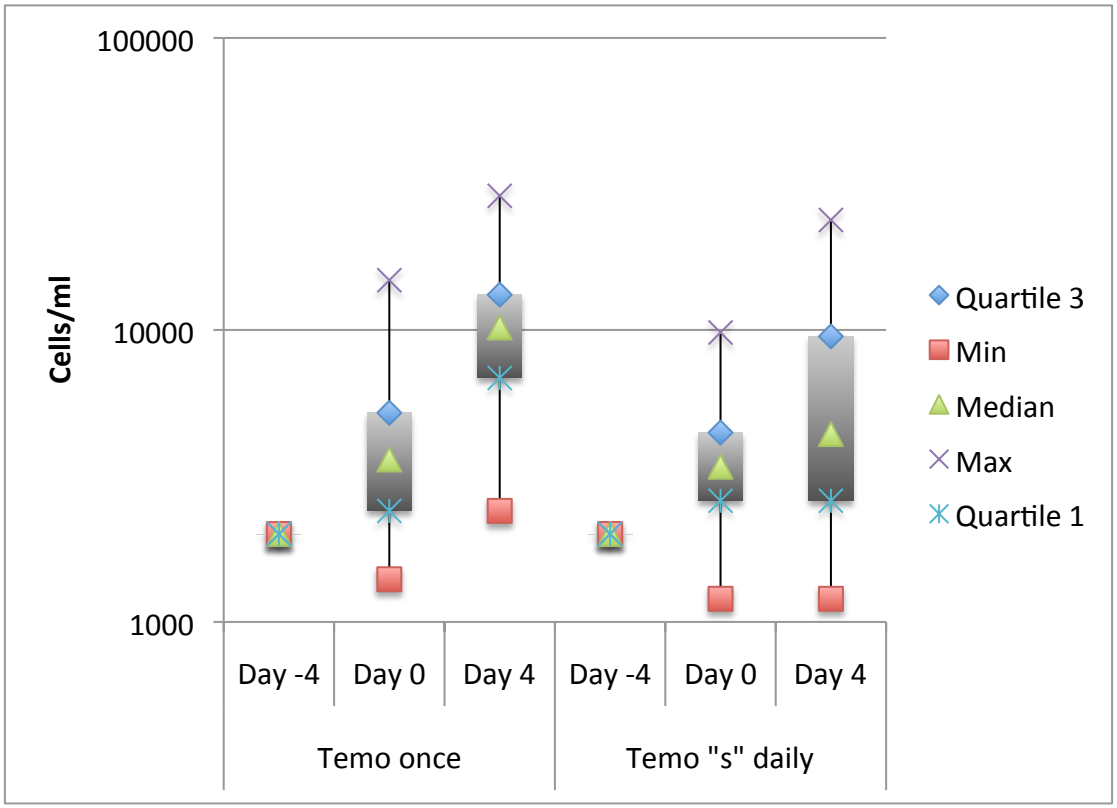

Figure 3-7 Comparison of Temozolomide single dose on non-synchronised versus Temozolomide daily dose on synchronised culture cells after four days of incubation. Temo once: single dose of Temozolomide on non-synchronised cells; Temo "s" daily: daily dose of Temozolomide on synchronised cells.

Based on these results there was significant difference between the effect caused by cell synchronisation and frequency of drug application to Temozolomide's kill efficacy when comparing single dose on non–synchronised cells to daily application on synchronised cells. The above was obvious from day 2 of incubation.

Table 3-7 Summary of p values between the different datasets.

	T once	T "s" once	T daily	T "s" daily	Cis once	Car once
T "s" once	0.088					
T daily	***<0.001***	0.139				
T "s" daily	**<0.05**	0.313	0.893			
Cis once	***<0.001***	***<0.001***	***0.001***	***<0.001***		
Car once	**<0.05**	**<0.05**	***<0.001***	***0.001***	***<0.001***	
Medium	**<0.05**	***<0.001***	***<0.001***	***<0.001***	***<0.001***	0.088

T once: single dose of Temozolomide on non-synchronised cells; T "s" once: single dose of Temozolomide on synchronised cells. T daily: daily dose of Temozolomide on non-synchronised cells; T "s" daily: daily dose of Temozolomide on synchronised cells; Cis once: single dose of Cisplatin on synchronised cells; Car once: single dose of Carmustine on synchronised cells. Bold values indicate p value below 0.05. Red values in italics indicate p value equal or below 0.001.

The table above (Table 3-7) summarises the effect of the different drugs and their frequency of application to the synchronised and non-synchronised cell lines. Briefly:

- Daily application of Temozolomide on non-synchronised cells was better than a single dose.
- Daily application of Temozolomide to non-synchronised cells had similar effect to daily application of Temozolomide on synchronised cells.
- Single dose of Temozolomide to non-synchronised cells had similar effect to a single dose of Temozolomide on synchronised cells.
- Daily application of Temozolomide to non-synchronised cells had similar effect to a single dose of Temozolomide on synchronised cells.
- Daily application of Temozolomide on synchronised cells was better than single dose of Temozolomide on non-synchronised cells.
- Frequency of application of Temozolomide did not change the effect of Temozolomide on synchronised cells.
- Single application of Cisplatin exhibited a better kill effect than Temozolomide regardless of Temozolomide's frequency of application and cell synchronisation.

- Cisplatin and Temozolomide exhibited a better kill effect than a single dose of Carmustine.
- Carmustine did not produce a significant kill effect, as it was no different to the medium.
- Temozolomide and Cisplatin had a significantly better kill effect than the medium.

3.6 Discussion

This study investigates the reasons that lead to Temozolomide's reduced efficiency when used in chemosensitivity studies. Temozolomide's suboptimal performance *in vitro* was also compared to that of other drugs used in the treatment of malignant gliomas, nominally Carmustine and Cisplatin. The results from this work showed that the frequency of application on the non-synchronised cell lines was a contributing factor to Temozolomide's efficiency. The daily application of Temozolomide gave a significantly better cell kill compared to a single dose at the onset of the experiment. This finding is in accordance with previously published studies [179], [180].

It is likely that the observed improvement of Temozolomide's performance when it was administered daily was due to the four-fold increase of the amount of drug used compared to the single dose (80 μg vs. 20 μg). A fair comparison would have been the administration of the same total amount to both populations but at different frequencies (daily versus once). Another possible explanation for the frequency of application effect observed is Temozolomide's short half-life. It is well known from Temozolomide's pharmacokinetics that its active component is very rapidly degraded to the inactive metabolite before it gets removed from the blood circulation. The whole process takes up to a few seconds to complete. It is reasonable to assume that a similar fate for Temozolomide is also taking place during its use in *in vitro* experiments. Single application of Temozolomide in cell cultures might have been rapidly degraded before it could have a measurable effect to the cell population.

As Temozolomide causes methylation and alkylation of DNA, it is predominantly effective during the DNA replication phase of the cell cycle also known as the "S" phase. Based on that, it is likely that only the cells that are in "S" phase when the active Temozolomide is present would be susceptible to it.

Therefore, one way to improve the kill effect of the drug would have been to influence the "vulnerability" of the cell populations prior to their treatment with Temozolomide. In other words, synchronisation of the cell population and subsequent treatment with Temozolomide while the majority of them are on the "S" phase of the cell cycle should in theory have made them more susceptible to the drug. Adema and coworkers have confirmed this hypothesis by combining Temozolomide with drugs that cause cell cycle arrest in "S" phase. Using this method, they have observed a significant enhancement of the cytotoxic effect in the treated lung cancer cell lines [181].

Interestingly, not all findings of this present study concord with the above hypothesis and the previously published reports. More specifically, synchronisation of cell populations did not increase the kill effect of Temozolomide when compared to the non-synchronised population. A possible explanation for this observation is that, the effect of the cell synchronisation was masked by the stronger impact of Temozolomide's daily application. A longer exposure to daily application of Temozolomide on synchronised cells might have revealed a significant difference when compared to the non-synchronised ones as the four-day period might not have been enough. Similarly, synchronisation did not produce a significant difference when Temozolomide was applied as a single dose. As there was no masking effect caused by the frequency of application, there should have been a significant improvement to the kill effect of the synchronised compared to the non-synchronised population. Given the fact that there was a trend of a decreasing p value over the four-day period, one could speculate that a longer exposure could have produced the expected effect. Future experiments could clarify this hypothesis by allowing longer incubation periods.

Further support the above hypothesis that synchronisation is equally important to the frequency of application, is provided by the fact that when compared there was no significant difference between them. This suggests that both have an equally prominent effect. In addition, even from day 2 the daily Temozolomide dose on synchronised cells exhibited a strikingly better effect

when compared to the single dose of Temozolomide on non-synchronised ones. This demonstrates the beneficial impact of both cell synchronisation and frequency of dosing on the Temozolomide's kill efficiency.

Of the other two drugs tested, Cisplatin displayed a much stronger kill effect than any of the other drug used. Carmustine on the other hand, was not found to exhibit any significant kill effect. In fact its efficacy was comparable to the medium. Both observations did not come as a surprise and are in agreement with the reported literature. More specifically, Cisplatin has been reported to have a good kill effect both in malignant glioma primary cultures and cell lines [156, 157]. Carmustine on the other hand, has been reported as having variable effect with few cultures showing *in vitro* sensitivity [126, 127].

It is apparent from this study that both frequency of application and cell synchronisation could enhance the sensitivity of *in vitro* testing of chemotherapeutic agents. Such enhancements increase handling time and require specialised equipment. Given the added value that they provide to chemosensitivity testing they should be worth the investment.

4 BIOMARKERS IN GLIOMAS: CYTOKINES

4.1 Introduction

There has been growing interest in discovering biomarkers for malignant gliomas. A biomarker is a measurable substance, which can used to indicate a biological state. Such state can be a normal biological process, a disease or even a pharmacological response to treatment. Examples of commonly used biomarkers include Hb_1Ac for diabetes mellitus, blood pressure monitoring in hypertension, and chromosomal abnormalities in genetic syndromes.

Biomarkers are also widely used as part of disease monitoring in several common cancers, such as prostate cancer, ovarian cancer, hepatocellular cancer, to name a few. In recent years, several studies have identified potential candidate molecules as brain tumour biomarkers but so far very few of them have been used in clinical practice [113]. Amongst the most promising ones for clinical use are the Cytokines and Angiogenesis factors [114]. Cytokines are the main focus of this chapter whereas the Angiogenesis factors are expanded in Chapter 5.

Cytokines are small protein molecules that are secreted by cells of diverse embryological origin during both physiological and pathological metabolic pathways. They are signalling molecules that are used in intercellular communication, triggering inflammation or in response to infection. They can be classified as proteins, peptides or glycoproteins and include interleukins, lymphokines and cell signal molecules, such as tumour necrosis factor and the interferons. These molecules have been investigated as potential biomarkers for brain tumours as well as molecules for targeted therapy [182]; [183].

Virtually all nucleated cells, but especially endothelial cells, epithelial cells and macrophages are the main producers of cytokines and mainly interleukins. As

these cells are found everywhere in the body, interleukins have systemic rather than local effects. The ubiquitous production of the interleukins is one of the features that differentiate them from the hormones. Interleukins are produced in response to physiological processes of intercellular communication as well as in response to trauma or inflammation. As such they can be measured in the blood of healthy individuals.

There have been several studies investigating the "normal" range of inflammatory cytokines in healthy individuals. In the plasma of healthy volunteers, IL-6 concentration was found to be between 0.01 to 11.5 pg/mL with a median concentration of 1.46 pg/mL [183]. Similarly, in the serum of healthy individuals, IL-6 was also found to be in the 1 pg/mL range [184] [185] with slight increase during the menstrual cycle [186], and larger increase after surgery (30-430 pg/mL)[187].

Ferrucci and coworkers [188] have demonstrated that the concentrations of the interleukins increase with age in healthy individuals. They claimed that the older population has a constitutional "pro-inflammatory state" as well as a more profound cytokine response after a noxious stimulus. There are some studies that also report an age difference in the circulating levels of IL-6, C-reactive protein (CRP) and other inflammatory biomarkers both in serum and in plasma [189]. Ershler and coworkers reported that serum IL-6 levels are normally non detectable, but become so with increasing age. This observation supports the previous hypothesis of the age related abnormal regulation of IL-6 gene expression. They also speculated that IL-6 contributes to the pathogenesis of several age-associated diseases such as lymphoma, Alzheimer's disease, and osteoporosis [190, 191]. Other studies involving healthy individuals have also demonstrated that age is associated with higher baseline levels of inflammatory cytokines. Environmental factors (i.e. smoking, infections, obesity, genetic factors and declining function of sex hormones) as well as age-associated diseases were found to be contributing causes to this observation. However, the definition of "healthy status" in those studies is debatable [192, 193].

Krabbe and coworkers have extensively investigated the cause of the "pro-inflammatory state" of the older healthy individuals. They have measured the baseline levels of several cytokines between two age groups of healthy volunteers. The younger group had a mean age of 24 years whereas the older group had a mean age of 66 years. All volunteers were screened for disease and had negative past medical history and physical examination. In addition, none of the volunteers used any medication, nor did they have any febrile illness in the fortnight preceding the study. Blood screening showed normal white blood cell (WBC) count, WBC differential count, CRP, blood glucose levels, kidney function, liver function, and coagulation screen. All individuals had a normal electrocardiogram (ECG). Furthermore, all older volunteers underwent an exercise ECG that was found to be normal. The study concluded that the baseline cytokine load did not differ between the two age groups. However, the older age group produced a more rapid and longer lasting acute phase inflammatory response when compared to the younger age group. This observation could be caused by a primary impairment of the mechanisms that turn off the inflammatory response. Another possible explanation could be the accumulation of cardiovascular risk factors and diseases with aging. The baseline plasma levels reported in the Krabbe study as normal for Il-6, IL-8 and IL-10 were 0.156 pg/ml, 31.2 pg/ml, and 0.781 pg/ml respectively [194].

Other studies, which applied strict criteria for good health, adequate nutrition and absence of diseases have also failed to detect any significant difference in the production of IL-1 and IL-6 between young, middle-aged, and older participants [195]. Kim and coworkers have profiled a large number of cytokines in serum of young (below 45 years) and elderly (over 65 years) healthy individuals using a multiplexed bead-based immunoassay. The serum levels of EGF, FGF-2, Flt-3L, INF- a2, INF-g, IL-10, IL-15, IL-17, IL-1b, IL-2, IL-6, IL-8, IP-10, MIP-1b, PDGF-AA, TNF-a, and VEGF that they reported did not show any significant difference between the two age groups. Participants of the older group had significantly higher systolic blood pressure (SBP), total cholesterol, triglyceride (TG), serum albumin, serum blood urea nitrogen (BUN), and serum creatinine levels. The serum circulating levels (pg/ml) which they reported as

normal for IL-6, IL-8 and IL-10 were: 2.91 (± 6.45), 23.99 (± 29.7) and 1.32 (± 3.06) respectively for the under 45, and 2.57 (± 5.22), 27.6 (±43.9) and 1.58 (± 6.17) for the over 65 [196].

Togo and coworkers tried to understand if there is a circadian rhythm to the secretion of these molecules. They demonstrated that the concentrations of the inflammatory cytokines varied during the sleep-awake cycle. Although their concentrations were uniformly low, there were bursts of secretion, which occurred during nighttime. The circulating plasma concentrations (pg/ml) that they reported as normal for healthy individuals were: 16.8 (±15.3) awake and 12.2 (±11.5) asleep for IL-6; 11.8 (±5.4) awake and 12.5 (±6.4) asleep for IL-8; and 0.95 (±0.38) awake and 0.84 (±0.38) asleep for IL-10 [197].

In addition to the cytokine producing cells mentioned above, there is accumulating evidence that certain cells of the Central Nervous System (CNS) also secrete several cytokines. These cell types include choroid plexus, ependymal cells, endothelial cells of the Blood Brain Barrier (BBB), microglia, neurons and astrocytes [198-200]. Follow up studies will shed light to the extent of the contribution of these cell types to the total concentration of circulating cytokines.

4.2 Aims

This study explores the feasibility of collecting serum and plasma samples from patients and healthy volunteers (controls) pre- and post-operatively. In addition it investigates the use of cytokines IL-6, IL-8 and IL-10 as potential biomarkers for malignant brain tumours. Furthermore, it scrutinises the effect of parameters such as the presence of brain tumour, the operative stage, the age and the gender to the serum levels of these cytokines.

4.3 Materials and methods

Serum samples were collected from 36 patients and 36 controls immediately pre-operatively and within 24 hours after the operation. Control patients were defined as patients treated surgically under a general anaesthetic in Preston's Neurosurgical Unit for benign disease (degenerative spinal disease such as disc protrusion or ligamentous thickening), with no past medical history including no known malignancy. None of the controls were on regular medications and in particular steroids of any form. The concentrations of 8 cytokines were measured using the Bio-Plex Pro Cytokines Assay (Bio-Rad) according to manufacturer's instructions [201]. The protocol is briefly outlined below.

4.3.1 Multiplex bead-based immunoassay

Simultaneous measurement of serum concentrations of granulocyte-monocyte colony-stimulating factor (GM-CSF), interferon γ (INF-γ), IL-10, IL-8, IL-6, IL- 4, IL-2, and Tumour necrosis factor- α (TNF-α), was performed using commercially available multiplex bead-based sandwich immunoassay kit (Bioplex) as per the manufacturer's instructions. Briefly, serum samples (50 µl per well) or standards (50 µl per well) were incubated with 50 µl of the pre-mixed bead sets in pre-wetted 96- well micro titer plates at room temperature for 30 minutes. After washing, 25 µl of the fluorescent detection antibody mixture was added for 30 min. After washing further 50 µl of streptavidin-phycoerythrin was added to each well for an additional 10 min at room temperature. After washing 125 microliters of assay buffer was added to each well. The plate was shaken at 1100rpm for 30 sec at room temperature prior to placing it into the reader. A range of 3.2-10,000 pg/mL recombinant full-length cytokine proteins was used to establish standard curves and to maximize the sensitivity and dynamic range of the assay. Cytokine levels were determined using a Bio-Plex 200 System (Bio-Rad Laboratories INC), and the Bio-Plex

Manager software was used for data acquisition and analysis. The data are reported as relative median fluorescent intensities [196].

4.4 Results

4.4.1 Biomarker analysis

Eight biomarkers were measured using the Bio-Plex Pro Cytokines Assay (Bio-Rad Laboratories Inc.). Of these, 3 cytokines (IL-6, IL-8 and IL-10) were the most significant ones. The raw measurements from these three biomarkers are summarized in the Appendix 4.

Outlier removal

Some of the measured concentrations were significantly outside of the physiological range and therefore the datasets were examined for potential outliers. Outlier removal was done using the method commonly referred to as the Quartile or Fourth-Spread method [202]. Essentially, it involves the identification of the boundaries of each of the quartiles in the data set. The difference between the 75th and 25th percentiles is the fourth spread (fs). The Upper and Lower Outlier Boundaries are equal to 1.5 times the fourth spread above and below the median. Using the Fourth-Spread method the outliers were removed from the datasets prior to continuing with the analysis. Once the outliers were removed, the remaining data presented a more uniform spread as shown in the boxplot below (Figure 4-1).

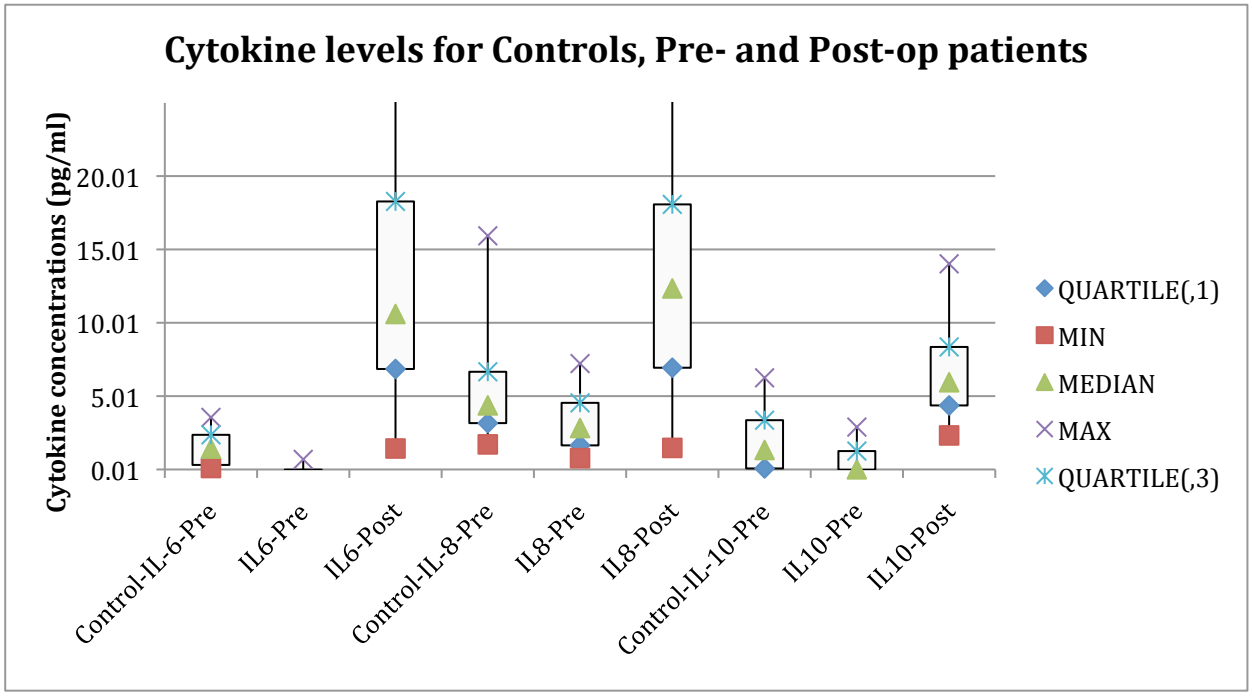

Figure 4-1*: Boxplot summarizing the pre- and post-operative serum concentration (pg/ml) of the three cytokines measured in the malignant patients and the controls (pre- only).*

Checking datasets for normality using Shapiro-Wilk Normality test

Before deciding which statistical method to be used for the data analysis, the data where checked whether they fit a normal distribution using the Shapiro-Wilk Normality test [203]. The null hypothesis for the Shapiro-Wilk test is that the data are normally distributed. If the chosen cut off score is 0.05 and the p-value is less than 0.05, then the null hypothesis that the data are normally distributed is rejected. If the p-value is greater than 0.05, then the null hypothesis has not been rejected and therefore the data are normally distributed.

According to this test almost none of the measurements derived from the Cytokines resemble a normal distribution and thus the Wilcoxon test was used [204]. The results from the Shapiro-Wilk normality test are summarised in Table 4-1.

Table 4-1*The Shapiro-Wilk scores and p-values as determined for the various datasets.*

Shapiro-Wilk Normality Test	
Cytokines data	
IL6-Pre	W = 1.98E-01, p-value = **<0.05**
IL6-Post	W = 9.02E-01, p-value = **<0.05**
IL8-Pre	W = 9.30E-01, p-value = **<0.05**
IL8-Post	W = 9.36E-01, p-value = 0.065
IL10-Pre	W = 7.24E-01, p-value = **<0.001**
IL10-Post	W = 9.41E-01, p-value = 0.115
Control-IL-6-Pre	W = 8.67E-01, p-value = **<0.05**
Control-IL-8-Pre	W = 8.10E-01 p-value = **0.001**
Control-IL-10-Pre	W = 8.49E-01, p-value = **<0.001**

Values above the 0.05 cut off score suggest normal distribution of the respective datasets. Generally the Cytokine datasets do not appear to be distributed normally. Significant values are shown in bold.

In terms of evaluating statistical significance given a calculated p-value, both significance levels α=0.001 (conservative) and α=0.05 (widely used) were considered. If a test of significance gives a p-value lower than the significance level α, the null hypothesis is rejected and the results are considered as 'statistically significant'.

Statistical package

The statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.15.1) running under Windows Vista OS with 2GB RAM [148]. All the statistical tests used here were included in the R Stats package.

4.4.2 Cytokines analysis

The results of the analysis of the cytokine IL-6, IL-8 and IL-10 are summarised in the table below (Table 4-2*)*. A more detailed description of the results is provided in the sections below.

Table 4-2 ***Summary of the p-values as resulted from the analysis of the cytokine levels (IL-6, IL-8, IL-10) across the different datasets. Significant values are shown in bold.***

	Age group	Cytokines		
		IL-6	IL-8	IL-10
Controls *vs.* Pre-op Patients	0-100	**<0.001**	**<0.05**	**<0.001**
Controls *vs.* Pre-op Patients	0-39	0.128	1.000	0.128
Controls *vs.* Pre-op Patients	40-69	**<0.001**	**<0.05**	**<0.001**
Controls *vs.* Pre-op Patients	70-100	**<0.05**	0.431	**<0.05**
Pre-op *vs.* Post-op Patients	0-100	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op Patients	0-39	0.100	0.629	0.100
Pre-op *vs.* Post-op Patients	40-69	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op Patients	70-100	**<0.001**	**<0.001**	**<0.001**

Pre-operative cytokine level comparison between Controls and Patients (all age groups)

To determine whether the cytokine levels of patients with malignant glioma were different from the levels in patients with benign disease and no malignancy, the measurements were compared using the Wilcoxon test. The results of the test are shown on the first row of Table 4-2 and Figure 4-2, Figure 4-3 and Figure 4-4.

The levels of all three Cytokines were significantly different (IL-6, $P<0.001$; IL-8 and IL-10, $P<0.05$) between patients with malignant tumour and patients with benign disease prior to the operation.

As there was indeed a strong difference on the cytokine pre-operative levels between the Controls and the tumour patients, a closer investigation was undertaken into whether this trend was equally common across our arbitrary assigned age groups: 0-39, 40-69, 70-100 years.

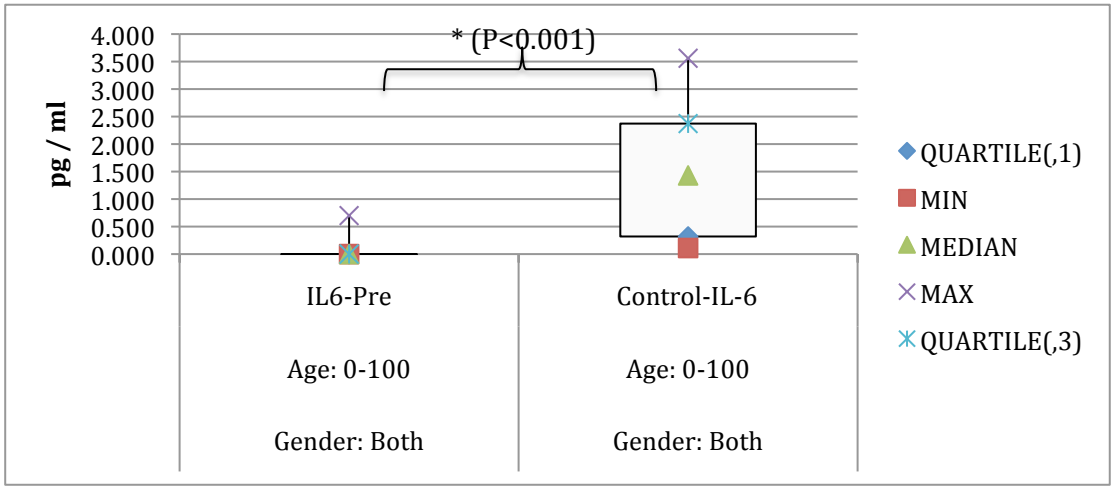

Figure 4-2 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients and the controls (pre- only) across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

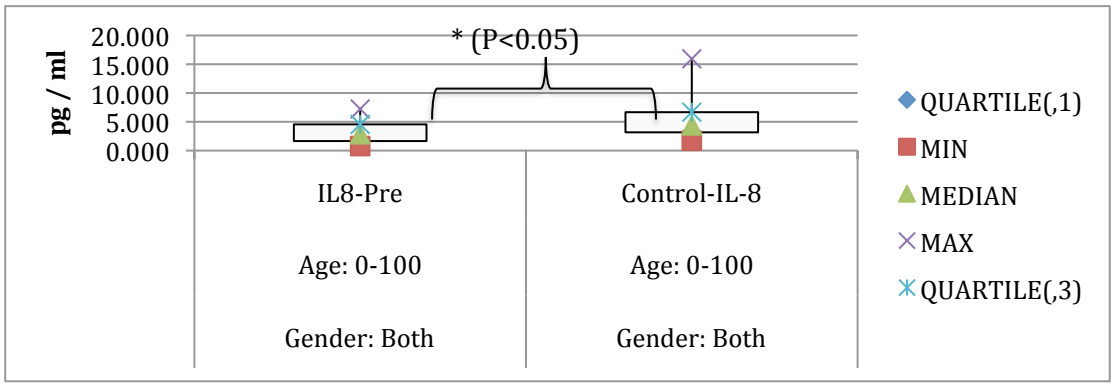

Figure 4-3 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients and the controls (pre- only) across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

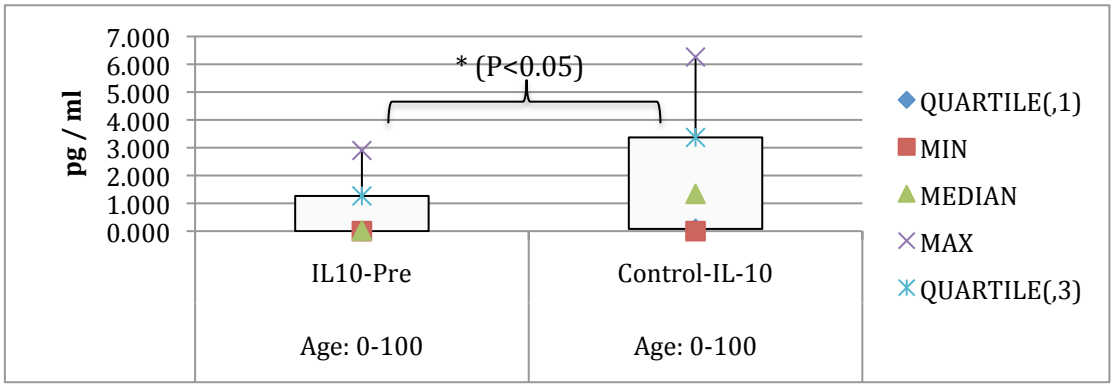

Figure 4-4 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients and the controls (pre- only) across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre-operative cytokine level comparison between Controls and Patients (age group 0-39 years)

The pre-operative cytokine levels assayed were tested in order to identify any statistical difference between the Controls and the tumour patients for the age group 0-39 years. The results from this comparison using Wilcoxon test are shown on the second row of Table 4-2 and Figure 4-5, Figure 4-6 and Figure 4-7. Based on them, none of three pre-operative Cytokine levels was significantly different (α=0.05) between the Controls and the tumour patients for the 0-39 years age group.

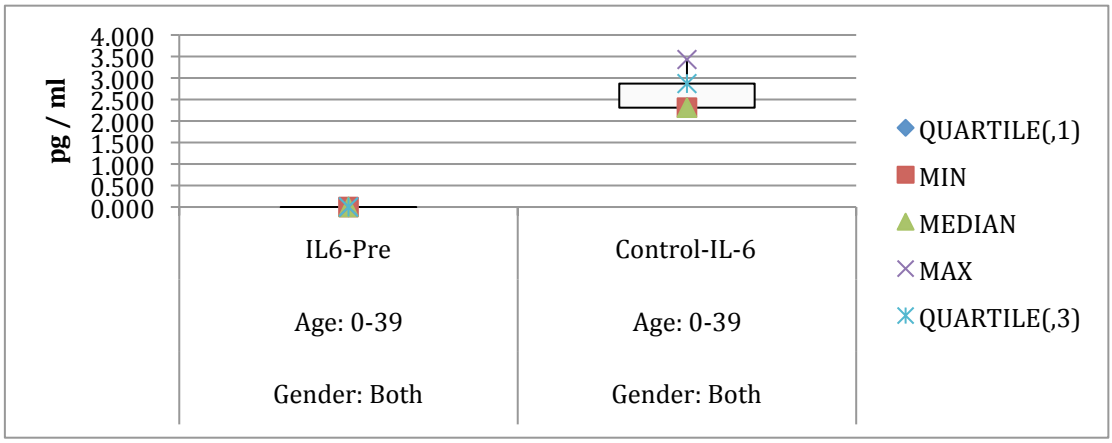

Figure 4-5 ***Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients and the controls (pre- only) in the 0-39 age group.***

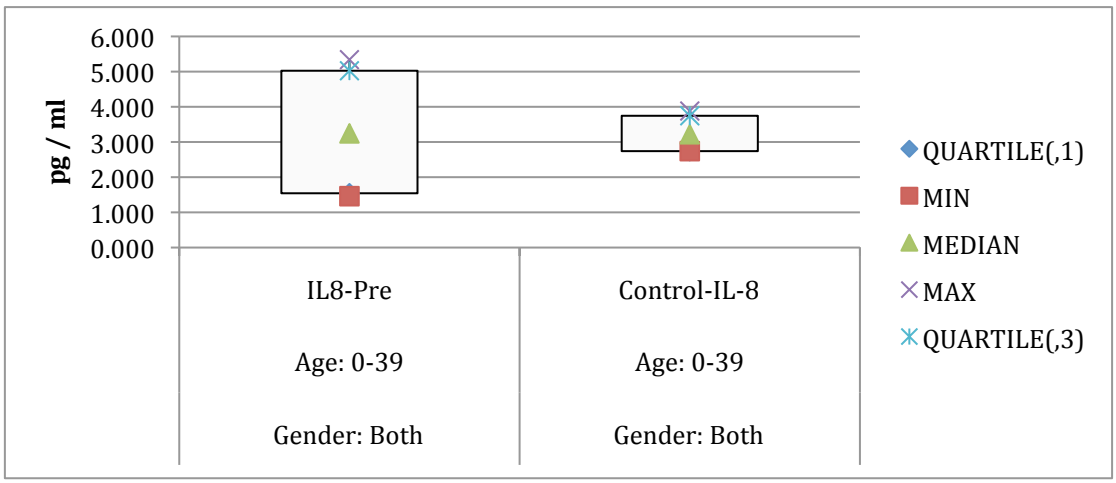

Figure 4-6 ***Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients and the controls (pre- only) in the 0-39 age group.***

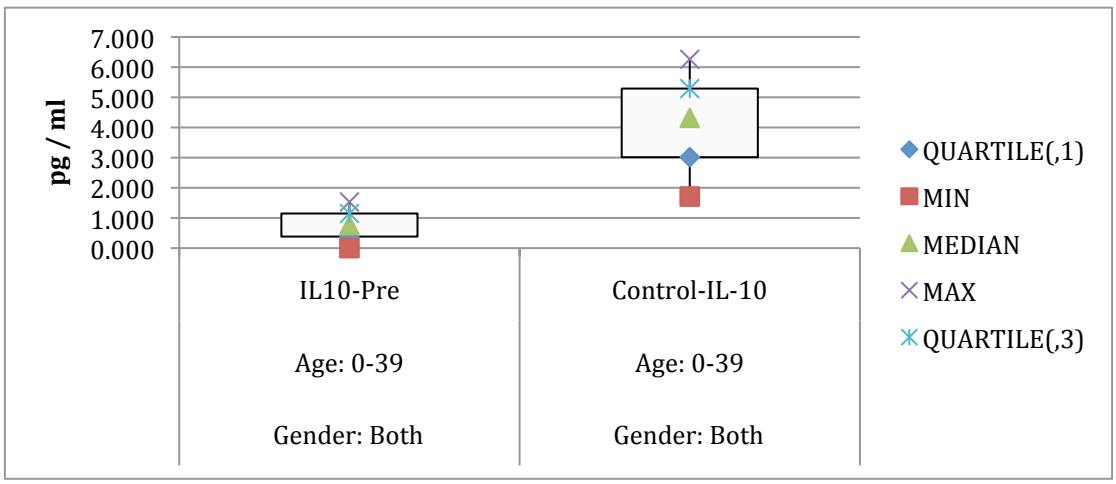

Figure 4-7 ***Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients and the controls (pre- only) in the 0-39 age group.***

Pre-operative cytokine level comparison between Controls and Patients (age group 40-69 years)

Similarly, the pre-operative cytokine levels between the Controls and the tumour patients for the age group 40-69 years were compared. The results from the Wilcoxon test for this age group are shown on the third row of Table 4-2 and Figure 4-8, Figure 4-9 and Figure 4-10.

Based on these results, the levels of all three pre-operative Cytokines were significantly different between the Controls and the tumour patients for this age group (IL-6 $P<0.001$; IL-8$P<0.05$; IL-10 $P<0.05$).

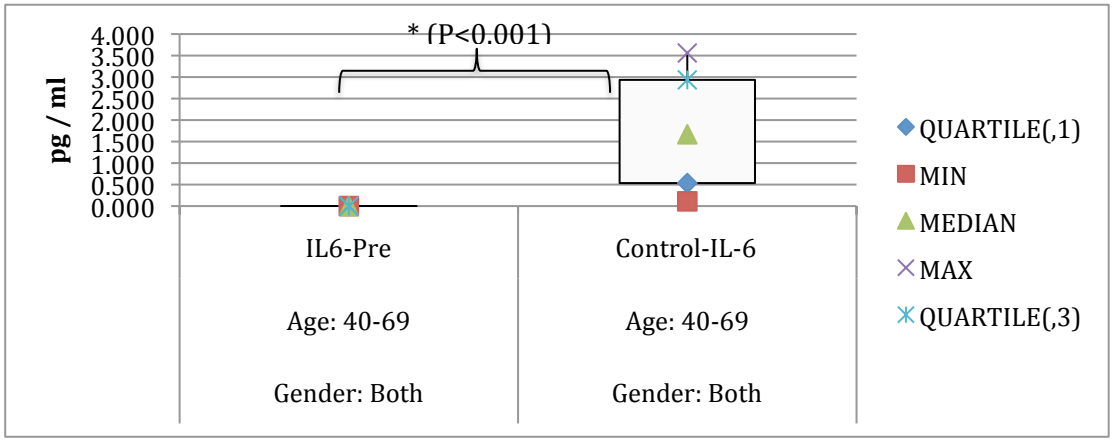

Figure 4-8 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients and the controls (pre- only) in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

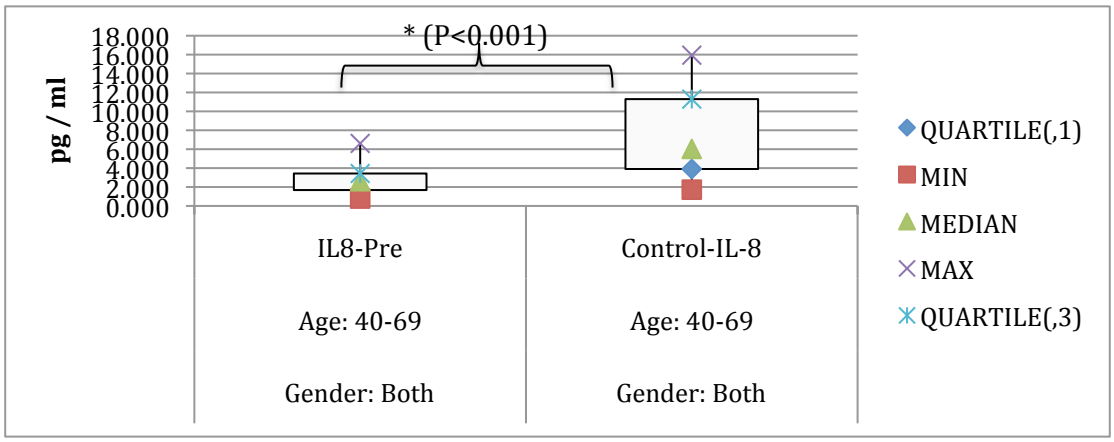

Figure 4-9 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients and the controls (pre- only) in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

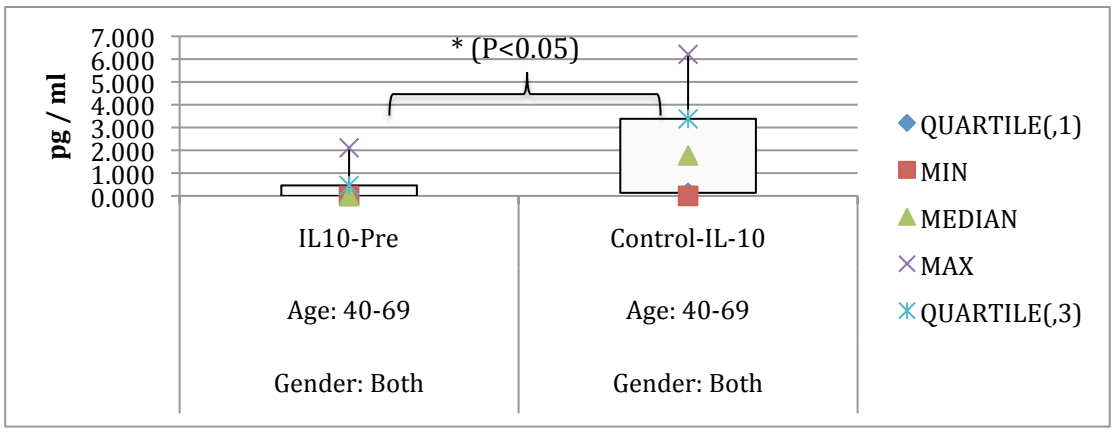

Figure 4-10 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients and the controls (pre- only) in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre-operative cytokine level comparison between Controls and Patients (age group 70-100 years)

The last age group to be tested for any difference in the pre-operative cytokine levels between the Controls and the tumour patients was the 70-100 years. The results from the Wilcoxon test for this age group are shown on the fourth row of Table 4-2 and Figure 4-11, Figure 4-12 and Figure 4-13.

The analysis has shown that only the pre-operative levels of IL-6 were significantly different between the Controls and the tumour patients for this age group ($P<0.001$).

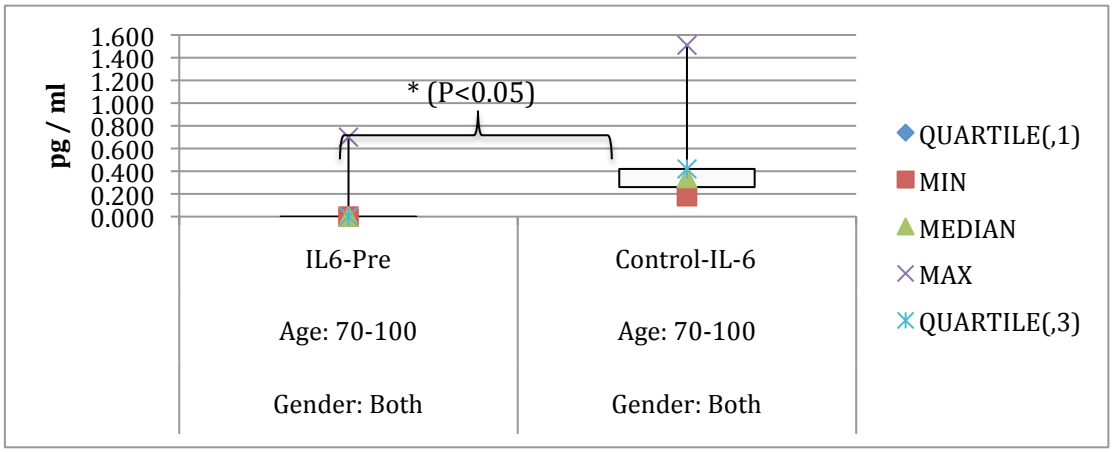

Figure 4-11 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients and the controls (pre- only) in the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

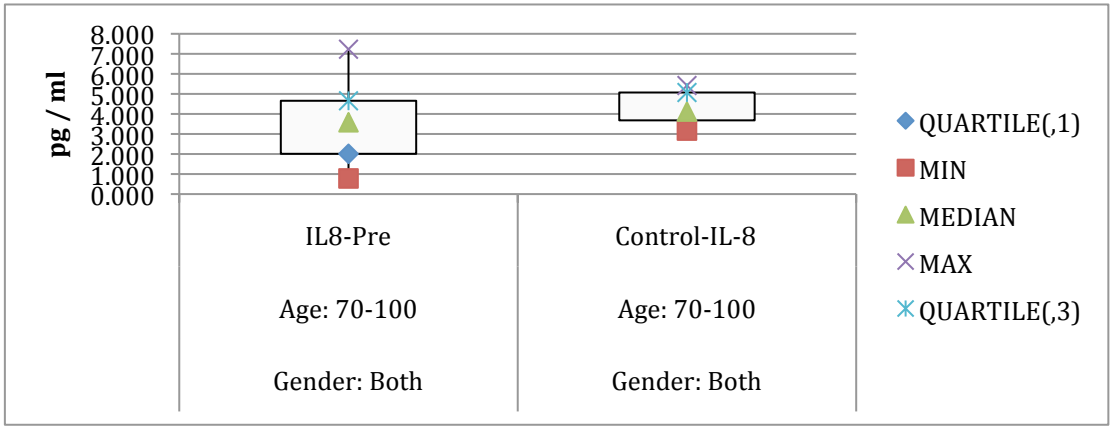

Figure 4-12 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients and the controls (pre- only) in the 70-100 age group.*

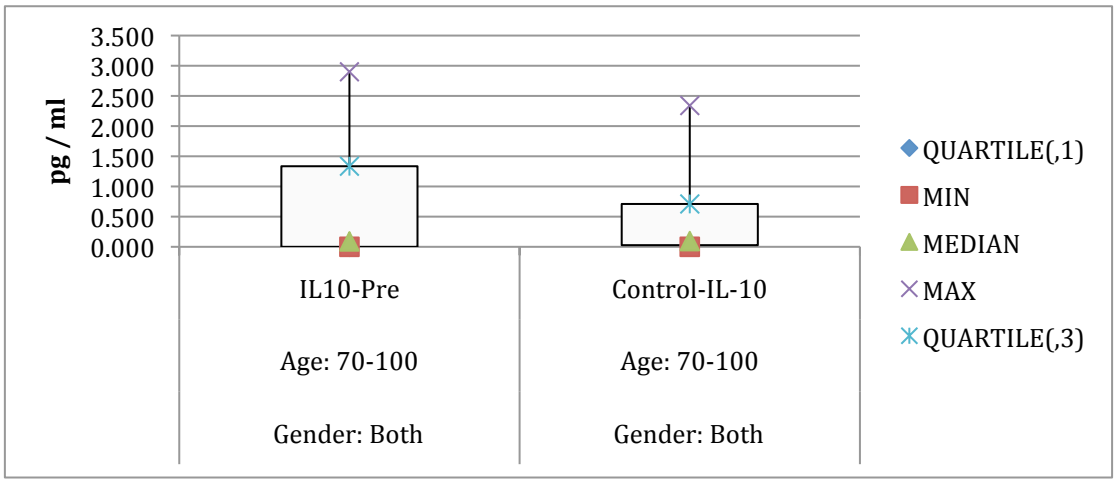

Figure 4-13 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients and the controls (pre- only) in the 70-100 age group.*

Pre- vs. post- operative Cytokine level comparison (all age groups)

One of the main questions of this study was whether there was a significant difference in the levels of patient's pre- and post-operative cytokines. To address this question, the Wilcoxon test was applied on the measured cytokine concentrations and the results of the analysis are shown on the fifth row of Table 4-2 and Figure 4-14, Figure 4-15 and Figure 4-16.

The levels of all three Cytokines were significantly different ($P<0.001$) on patients prior to and after their operation. Based on these findings, that there was indeed a strong difference on the cytokine levels pre- and post-operatively, the data were further analysed in order to check whether this trend was equally common across our arbitrary assigned age groups: 0-39, 40-69, 70-100 years.

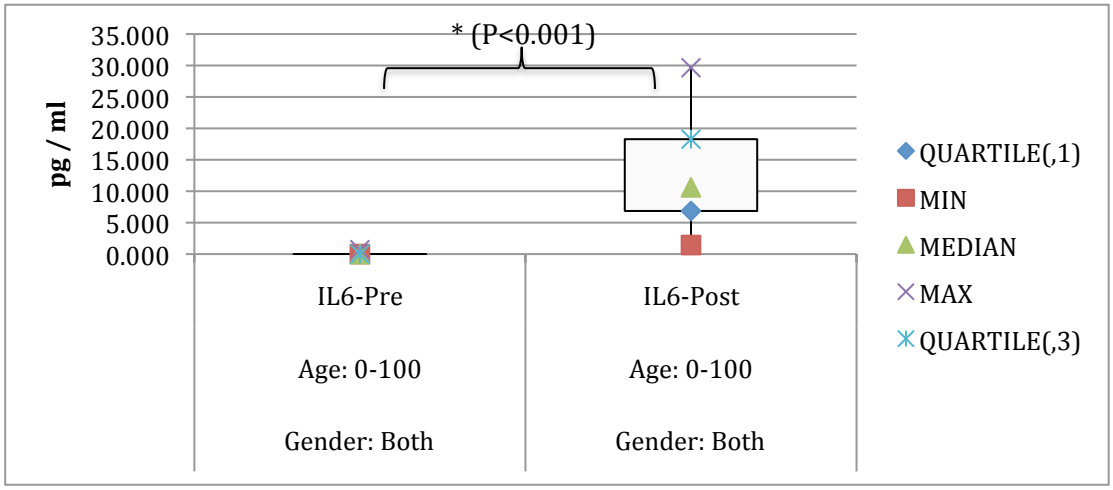

Figure 4-14 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients in all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

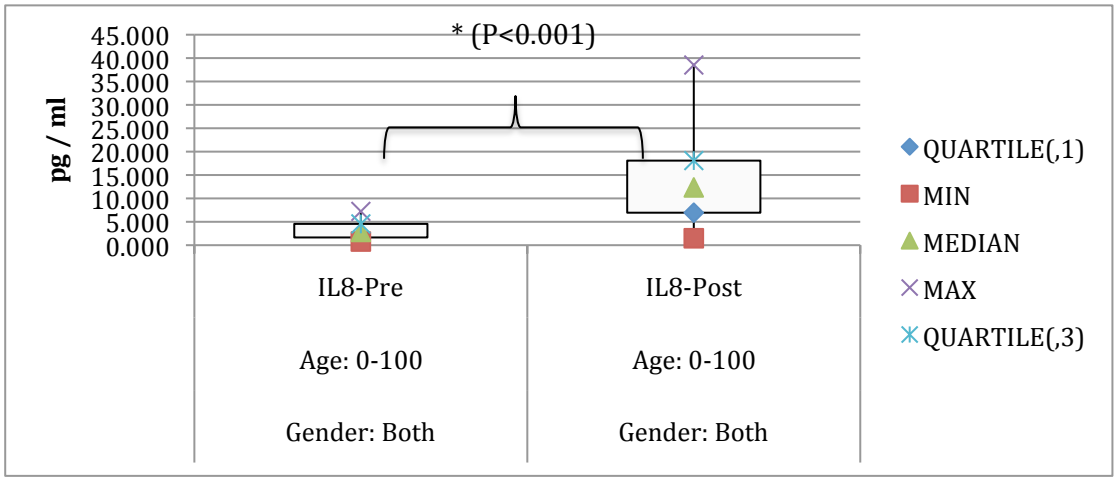

Figure 4-15 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients in all age groups.*

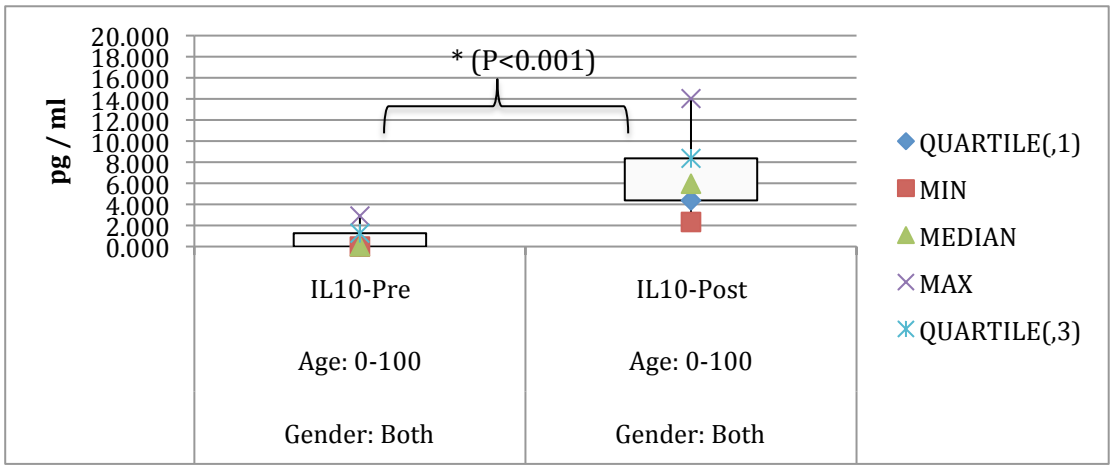

Figure 4-16 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients in all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre- vs. post- operative Cytokine level comparison (age group 0-39 years)

The pre- and post-operative cytokine levels were analysed further in order to identify a statistical difference between the samples for the age group 0-39 years. The results from this comparison using Wilcoxon test are shown on the sixth row of Table 4-2 and Figure 4-17, Figure 4-18 and Figure 4-19.

There was no significant difference (α=0.05) between the three Cytokine assayed on patients prior to and after the operation for the 0-39 years age group.

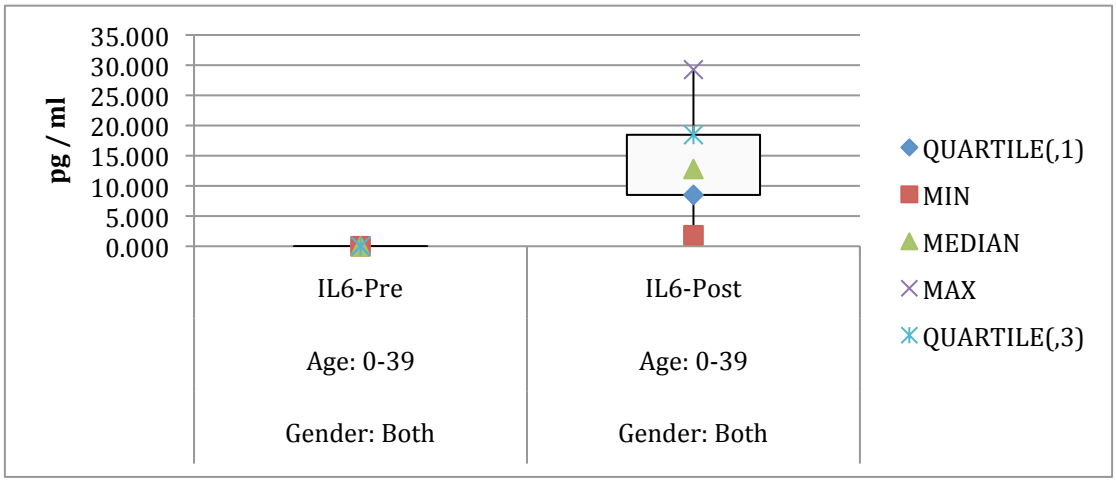

Figure 4-17 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients in the 0-39 age group.*

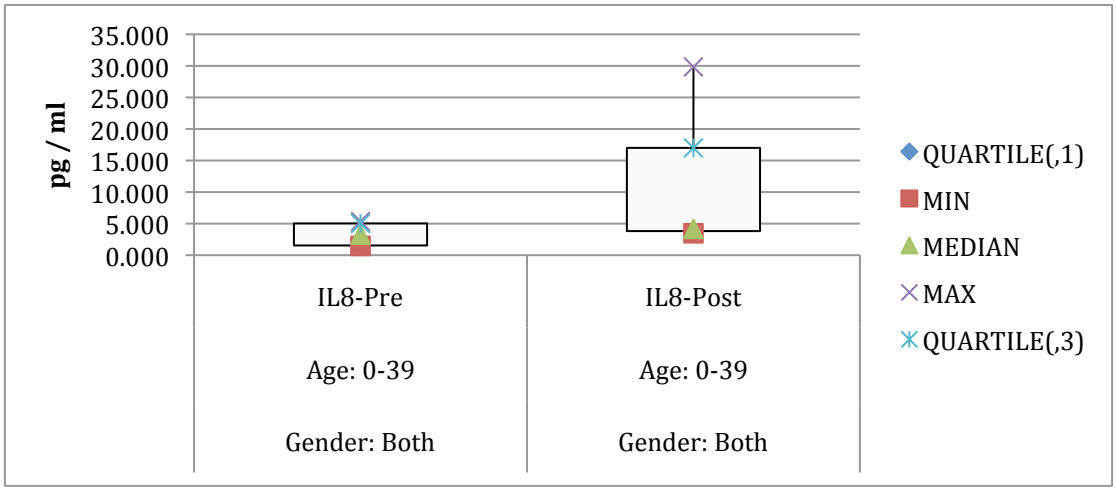

Figure 4-18 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients in the 0-39 age group.*

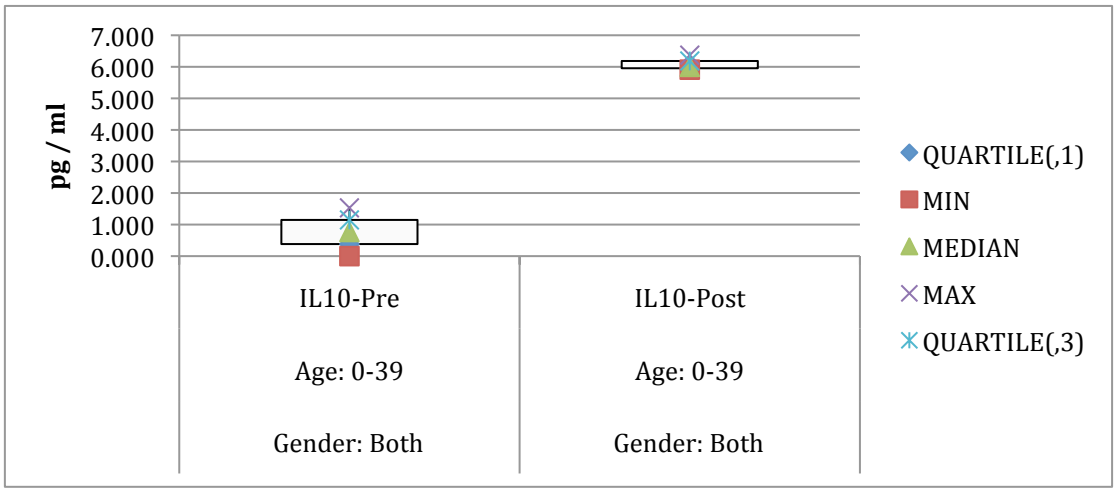

Figure 4-19 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients in the 0-39 age group.*

Pre- vs. post-operative Cytokine level comparison (age group 40-69 years)

Similarly, the levels of the cytokines between the pre- and post-operative samples for the age group 40-69 years were analysed to look for statistical difference. The results from the Wilcoxon test for this age group are shown on the seventh row of Table 4-2 and Figure 4-20, Figure 4-21 and Figure 4-22.

There was a significant difference in the levels of three Cytokines assayed ($P<0.001$) on patients prior to and after the operation for this age group.

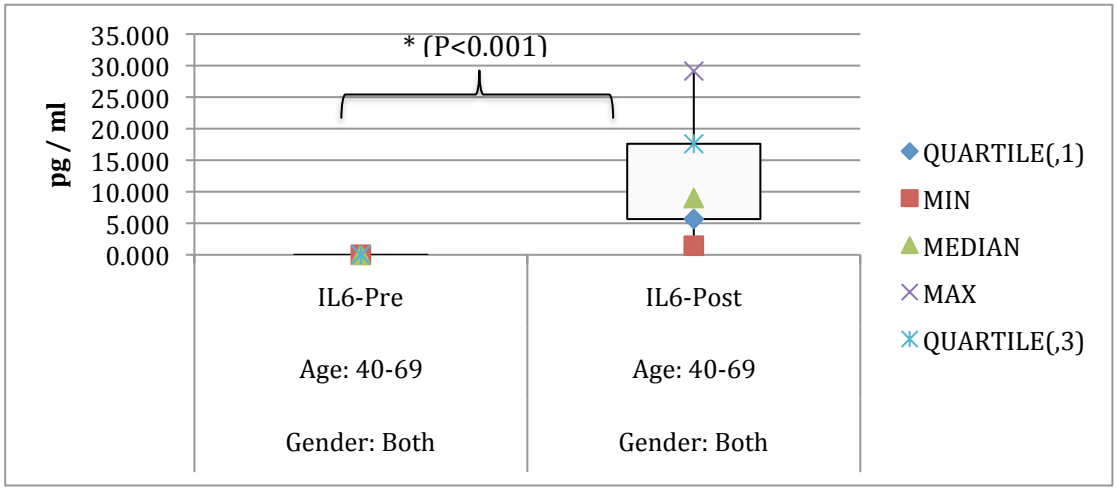

Figure 4-20 ***Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

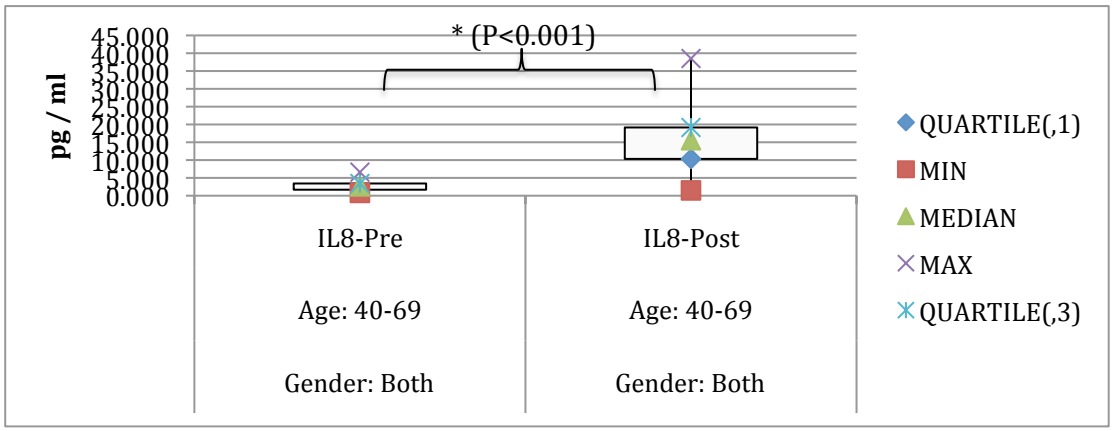

Figure 4-21 ***Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

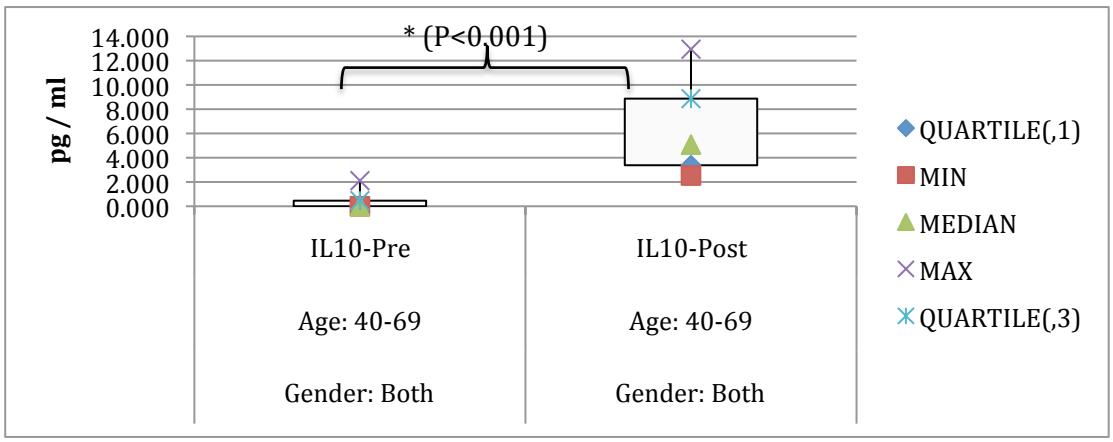

Figure 4-22*Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients in the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre- vs. post- operative Cytokine level comparison (age group 70-100 years)

The last age group to be tested for any difference in the cytokine levels pre- and post operatively was the 70-100 years. The results from the Wilcoxon test for this age group are shown on the eighth row of Table 4-2 and Figure 4-23, Figure 4-24 and Figure 4-25.

The analysis has shown that the levels of all three cytokines were significantly different ($P<0.001$) on patients prior to and after the operation for this age group.

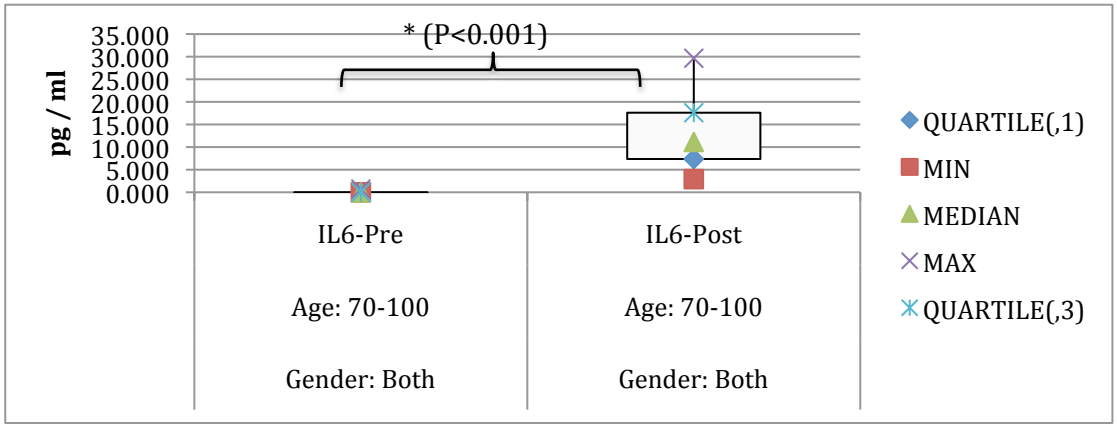

Figure 4-23 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients in the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

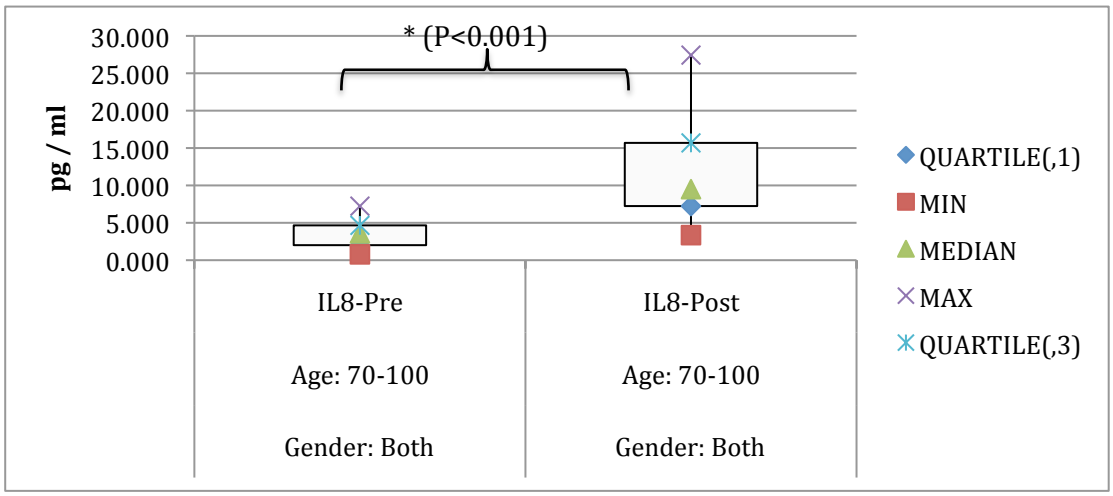

Figure 4-24 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients in the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

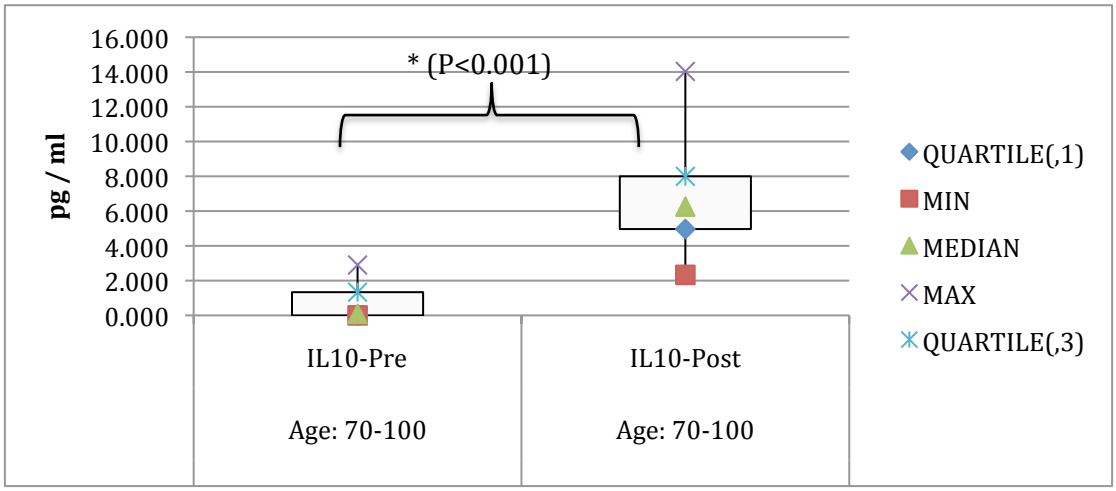

Figure 4-25 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients in the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Age group comparison within the pre- and the post-operation datasets

In the above analysis it has been established whether there is any difference in the cytokine levels for the different age groups between the pre- and post-operative samples and between the Controls and the pre-operative patient samples.

The next thing to look into is whether age plays a role on the levels of pre- and post-operative cytokines as well as on the Controls. Using the Wilcoxon test the levels of cytokines were compared between the three age groups in the pre- or post-operative datasets. The results are shown in Table 4-3, Table 4-4, Table 4-5 and Table 4-6 respectively.

Table 4-3 ***Comparison of the cytokine levels between the different age groups in the Control pre-operation datasets (p-values as determined by the Wilcoxon test). Significant values are shown in bold.***

	0-39 *vs.* 40-69	0-39 *vs.* 70-100	40-69 *vs.* 70-100
Control IL-6 (Pre-op)	0.350	**<0.05**	0.078
Control IL-8 (Pre-op)	0.078	0.176	0.335
Control IL-10 (Pre-op)	0.128	**<0.05**	0.221

Table 4-4 ***Comparison of the IL-6 levels between the different age groups in the pre- and the post-operation datasets***

IL-6 (Pre)	0-39	40-69	70-100	IL-6 (Post)
0-39		0.6608	0.8784	0-39
40-69	N/A		0.6475	40-69
70-100	0.8312	0.3156		70-100

p-values as determined by the Wilcoxon test. Significant values are shown in bold. White cells represent preoperative p values; light grey cells represent postoperative values. N/A means not enough data were available to allow the comparison.

Table 4-5 ***Comparison of the IL-8 levels between the different age groups in the pre- and the post-operation datasets.***

IL-8 (Pre)	0-39	40-69	70-100	IL-8 (Post)
0-39		0.4975	0.6107	0-39
40-69	0.7497		0.3162	40-69
70-100	1	0.3118		70-100

p-values as determined by the Wilcoxon test. Significant values are shown in bold. White cells represent preoperative p values; light grey cells represent postoperative values.

Table 4-6 ***Comparison of the IL-10 levels between the different age groups in the pre- and the post-operation datasets.***

IL-10 (Pre)	0-39	40-69	70-100	IL-10 (Post)
0-39		0.6107	0.8851	0-39
40-69	0.793		0.6114	40-69
70-100	0.9191	0.4334		70-100

p-values as determined by the Wilcoxon test. Significant values are shown in bold. White cells represent preoperative p values; light grey cells represent postoperative values.

Significant difference was observed only between the 0-39 years and 70-100 years age groups for the IL-6 and IL-10 on the Control samples ($P<0.05$).

No significant difference on the levels of the tested cytokines was observed between the different age groups either prior to or after the operation on the samples from Patients with glioblastoma ($\alpha=0.05$).

Effect of gender on pre- and post-operative cytokine levels

Finally, it was investigated whether gender had an effect on the pre- and post-operative cytokine levels as well as between the tumour Patients and the Controls. This was achieved by grouping the patients based on their gender. The cytokine levels of the different gender groups were compared using the Wilcoxon test. The results of the analysis are shown in Table 4-7 and Figure 4-26, Figure 4-27, Figure 4-28, Figure 4-29, Figure 4-30 and Figure 4-31.

Table 4-7 ***Comparison of pre- and post-operative levels of cytokines IL-6, IL-8 and IL-10 and gender (P values as determined by the Wilcoxon test). Significant values are shown in bold.***

		Cytokine		
		IL-6	**IL-8**	**IL-10**
All data *vs.* Male	Pre-op	0.752	0.501	0.758
	Post-op	0.533	0.818	0.722
All data *vs.* Female	Pre-op	0.577	0.387	0.629
	Post-op	0.388	0.755	0.517
Female *vs.* Male	Pre-op	0.477	0.185	0.495
	Post-op	0.204	0.646	0.387
Pre-op *vs.* Post-op	All Male	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op	All Female	**<0.001**	**<0.001**	**0.001**
Male Control *vs.* Male Patient	Pre-op	**<0.001**	**<0.05**	**<0.05**
Female Control *vs.* Female Patient	Pre-op	**<0.001**	**<0.05**	0.413
Male Control *vs.* Female Control	Pre-op	0.334	0.552	0.354

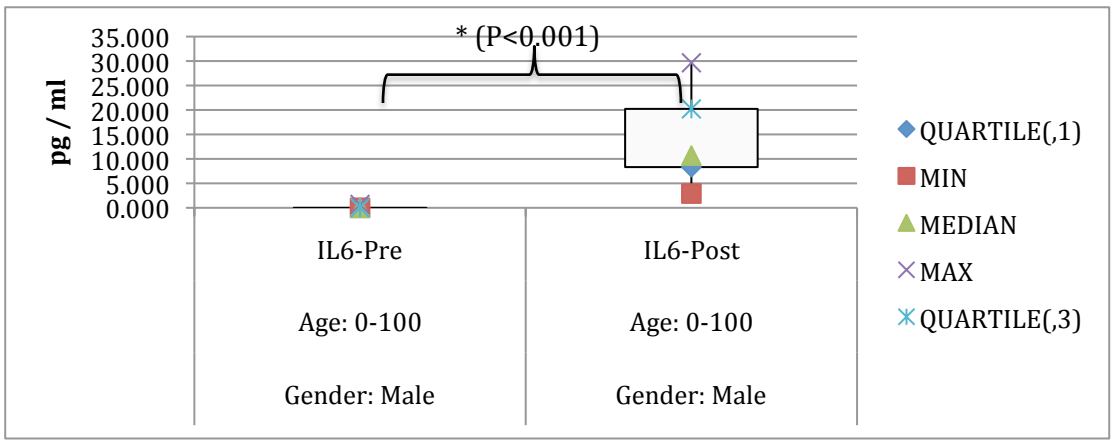

Figure 4-26 ***Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

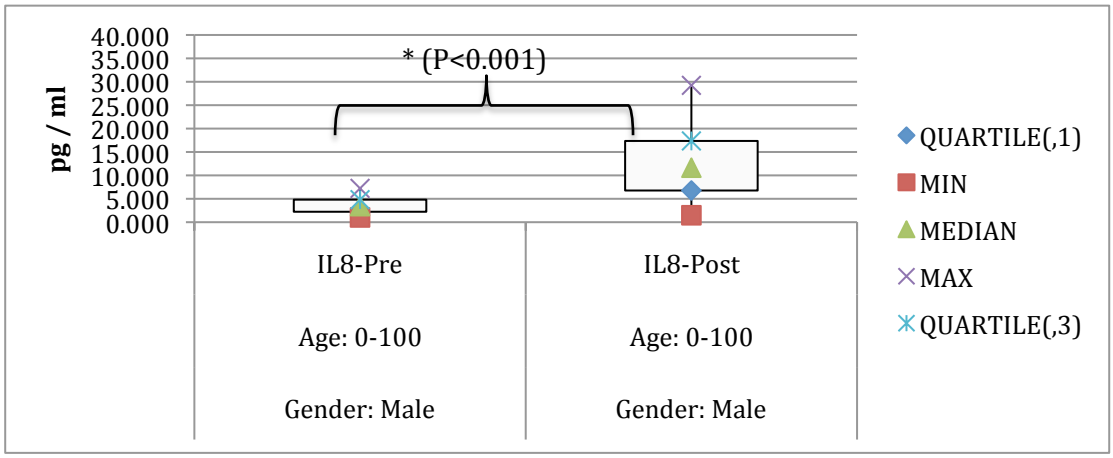

Figure 4-27 ***Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

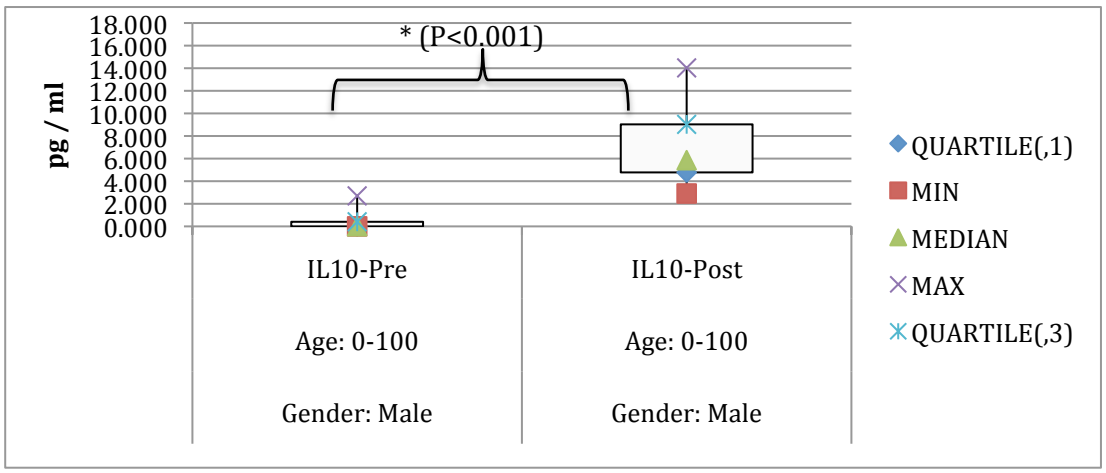

Figure 4-28 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

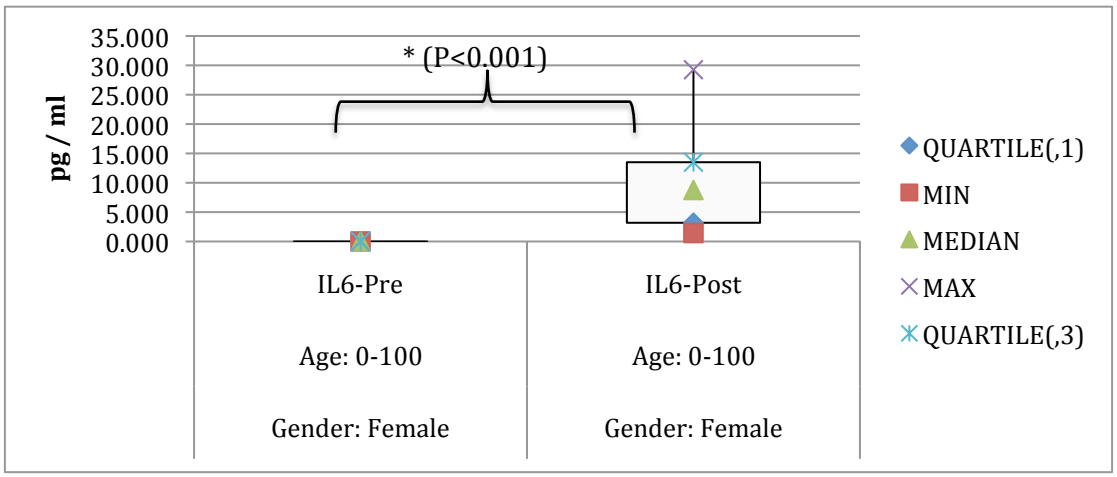

Figure 4-29 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-6 measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

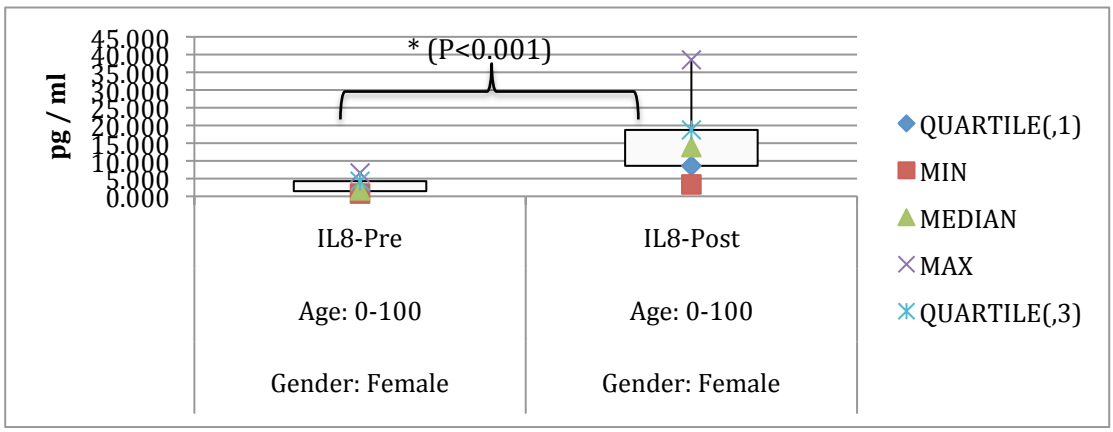

Figure 4-30 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-8 measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

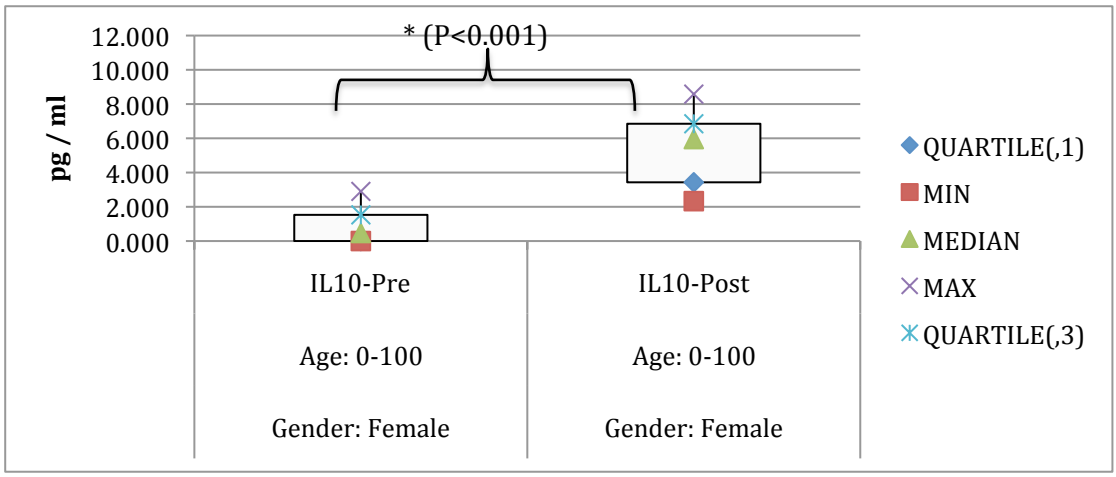

Figure 4-31 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of IL-10 measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

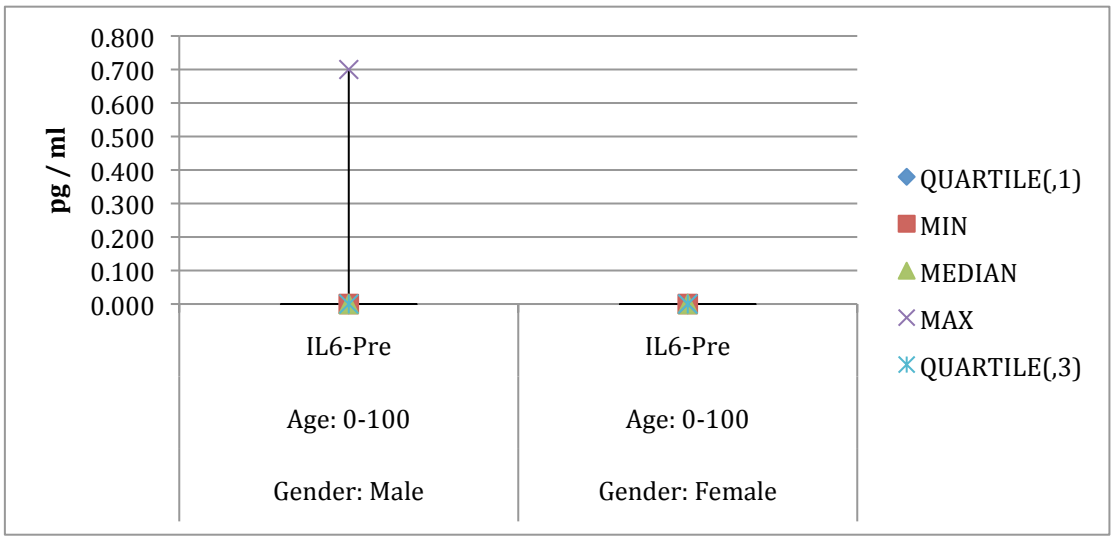

Figure 4-32 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients of each gender in all age groups.*

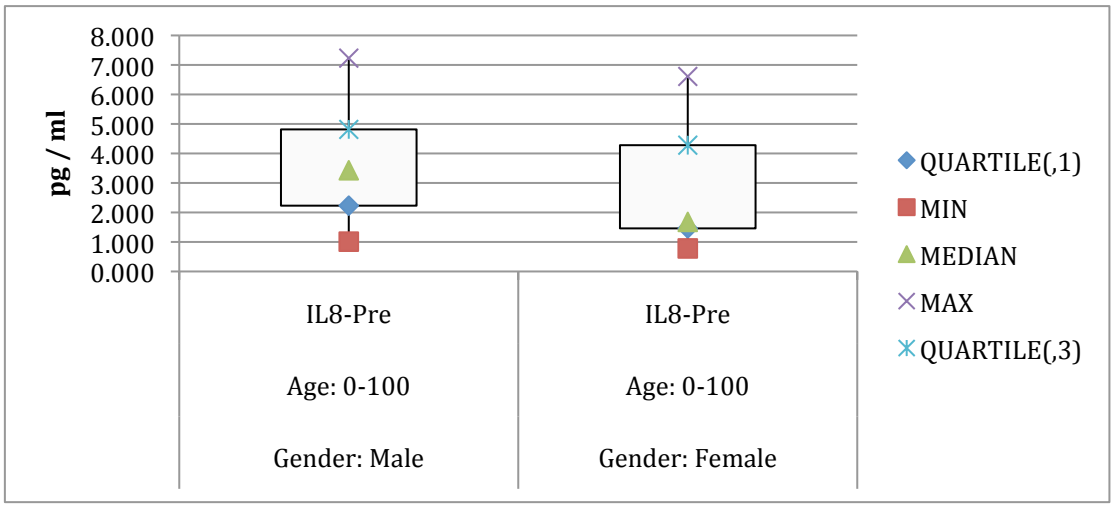

Figure 4-33 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients of each gender in all age groups.*

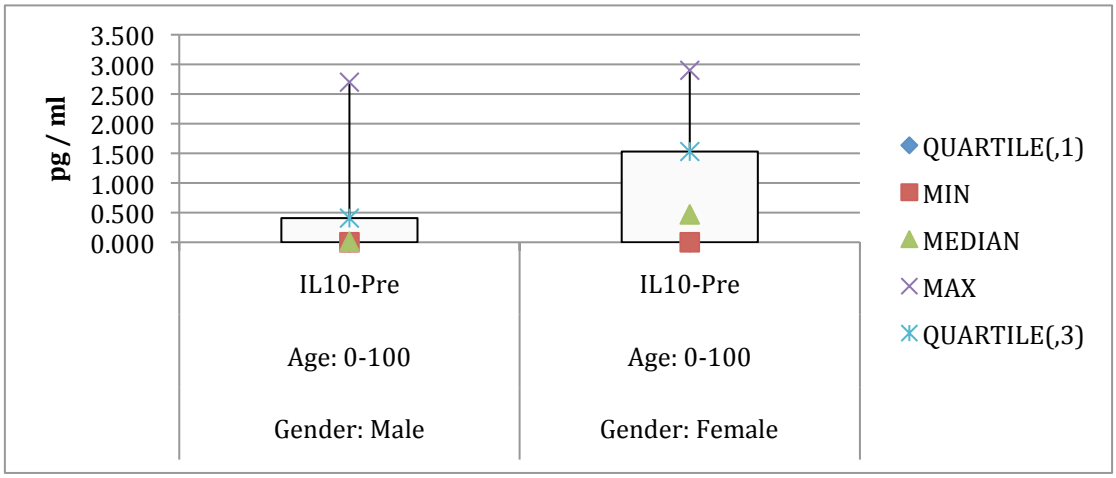

Figure 4-34 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients of each gender in all age groups.*

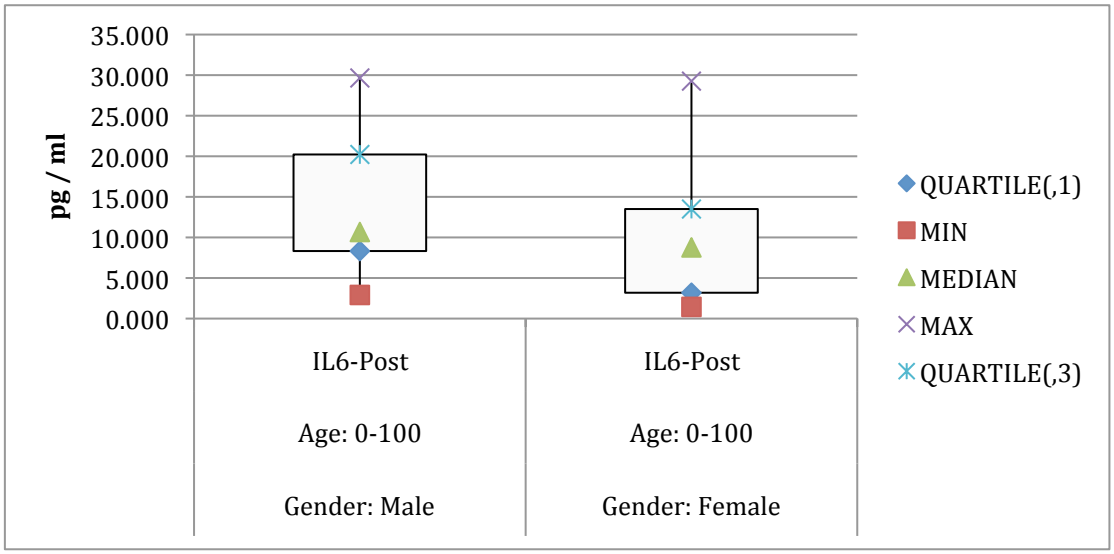

Figure 4-35 *Boxplot summarizing the post-operative serum concentration (pg/ml) of IL-6 measured in the malignant patients of each gender in all age groups.*

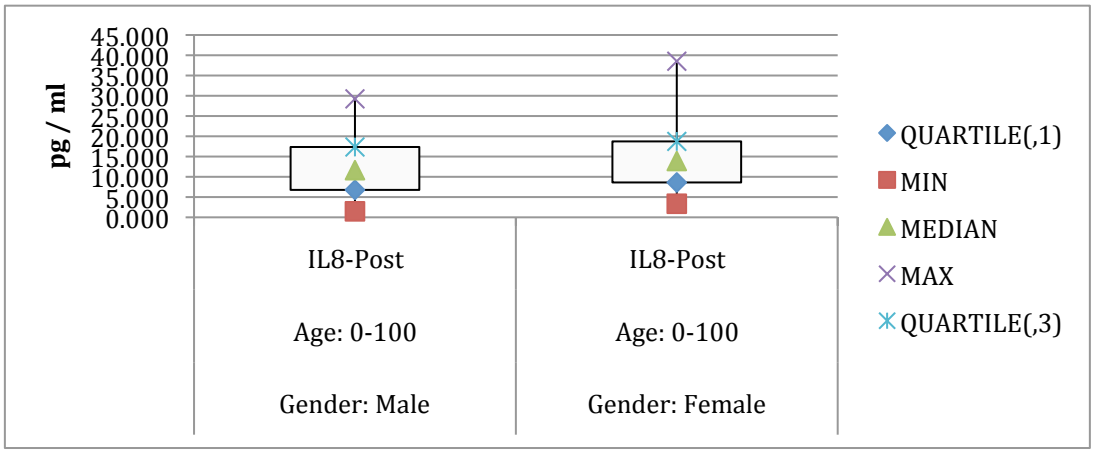

Figure 4-36 *Boxplot summarizing the post-operative serum concentration (pg/ml) of IL-8 measured in the malignant patients of each gender in the all age groups.*

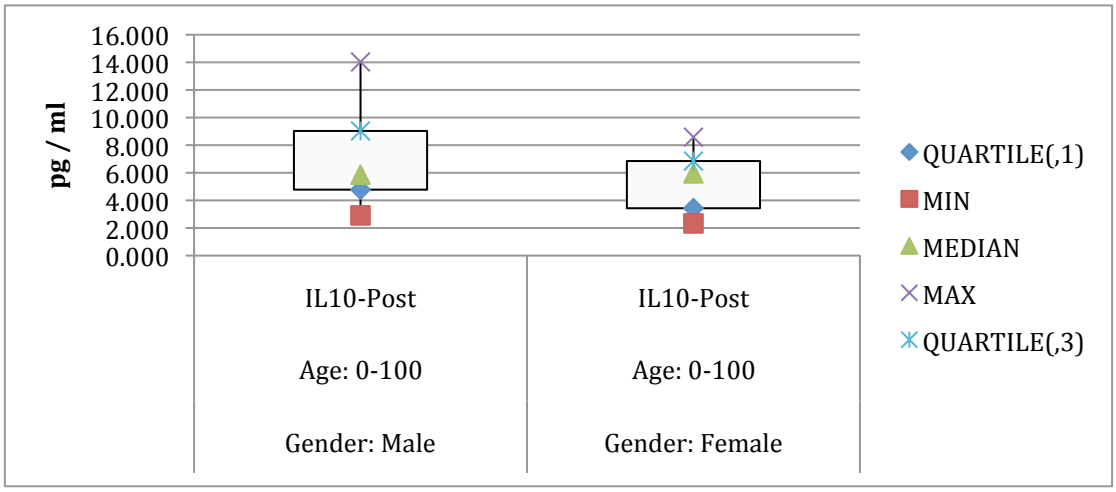

Figure 4-37 *Boxplot summarizing the post-operative serum concentration (pg/ml) of IL-10 measured in the malignant patients of each gender in all age groups.*

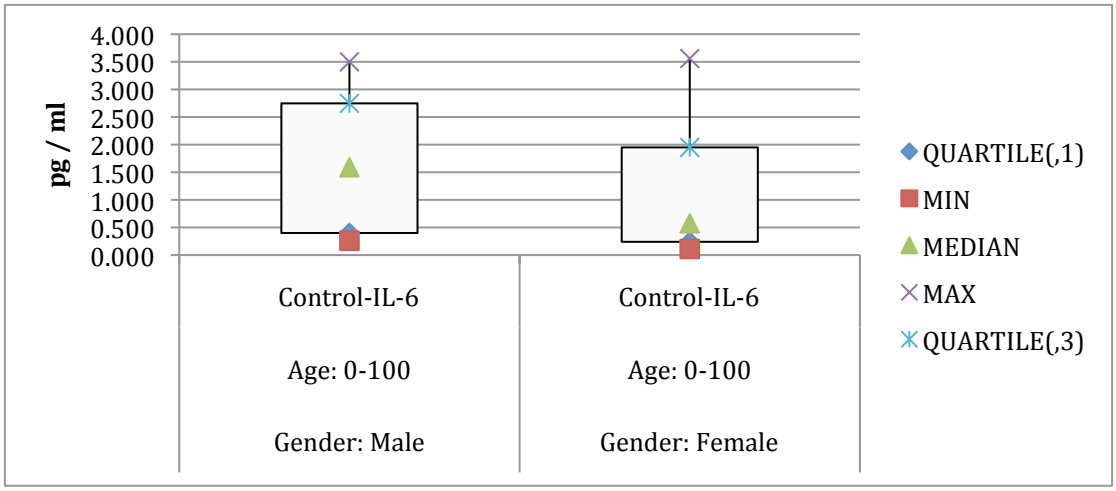

Figure 4-38 *Boxplot summarizing the serum concentration (pg/ml) of IL-6 measured in the controls (pre- only) of both genders in all age groups.*

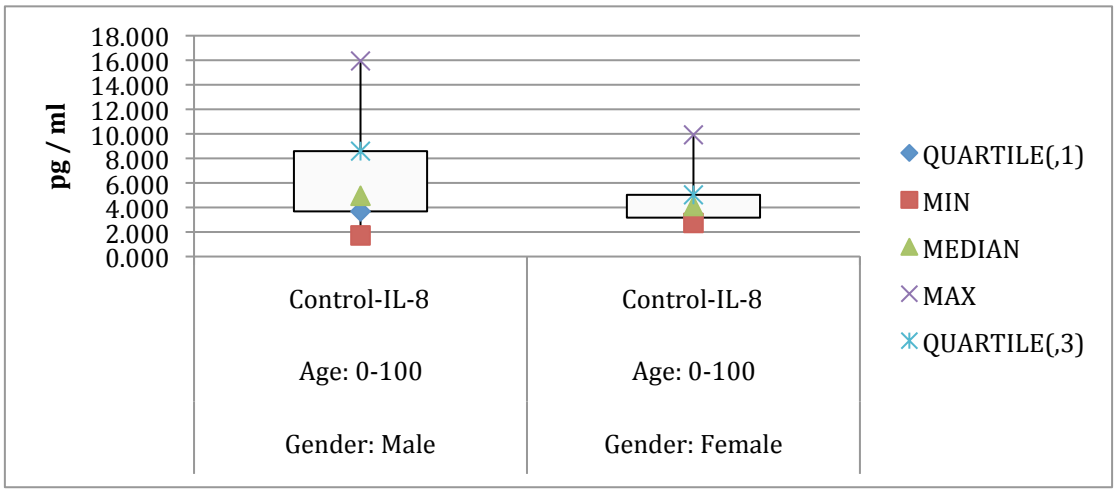

Figure 4-39 *Boxplot summarizing the serum concentration (pg/ml) of IL-8 measured in the controls (pre- only) of both genders in all age groups.*

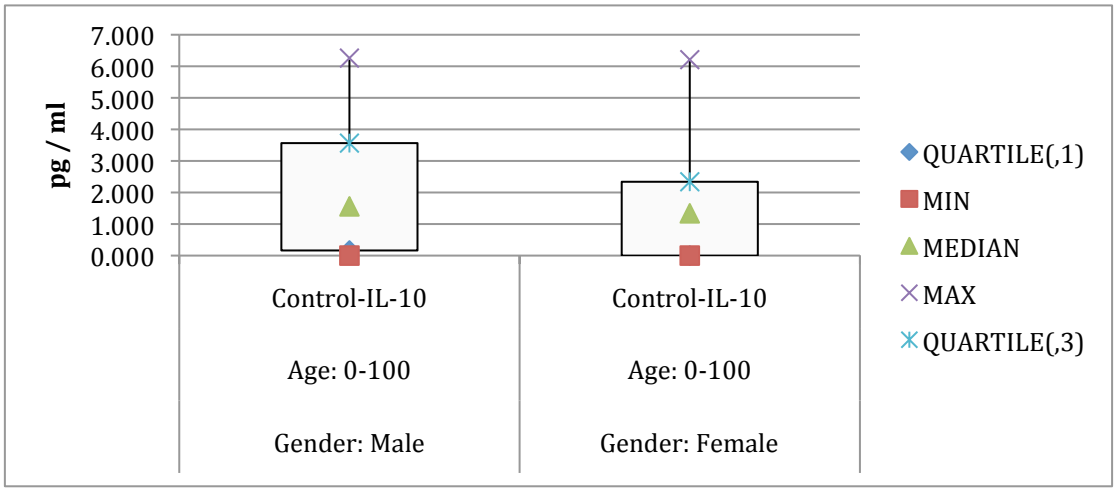

Figure 4-40 ***Boxplot summarizing the serum concentration (pg/ml) of IL-10 measured in the controls (pre- only) of both genders in all age groups.***

According to these results, neither the male nor the female group had displayed any significant difference against the combined dataset (males and females) as well as against the opposite gender (α=0.05). This suggests that gender does not seem to play a significant role for patients with glioblastoma multiforme. These observations held true for both gender groups regardless of their operative stage (pre- *vs.* post-) (Figure 4-32, Figure 4-33, Figure 4-34, Figure 4-35, Figure 4-36 and Figure 4-37). On the contrary, when the operative stage was compared against each other for each gender group, both male and female groups had shown significant difference for all three cytokines ($P<0.001$; All Female IL-10 <0.05).

Similarly to the results above, gender did not seem to have an effect in the preoperative Controls (α=0.05) either (see Male Control vs. Female Control in Table 4-7 as well as Figure 4-38, Figure 4-39 and Figure 4-40). However, with the only exception of IL-10 in females, both male and female Controls had significantly different cytokine levels from their equivalent patient samples (IL-6 $P<0.001$; IL-8 $P<0.05$). This observation confirms the significant difference observed previously between the Controls and the Patients when the male and female data were combined (Table 4-2).

4.5 Discussion

The serum concentrations of three cytokines were measured from patients with malignant gliomas as well as from controls affected by degenerative spinal disease. All three cytokines were found to be higher in the controls compared to the preop patients. In addition, the preoperative levels of all three cytokines were lower than those observed postoperatively. Age and gender did not play a role on the angiogenesis factor concentrations.

In this study, the concentrations observed in the serum of control individuals preoperatively were 1-2 pg/ml for IL-6, 4 pg/ml for IL-8 and 1 pg/ml for IL-10. These concentrations are slightly higher when compared to the values reported in the literature. This finding is not surprising as all healthy controls were affected by degenerative spinal disease. It is well known that degenerative spinal disease both in the form of disc protrusion as well as ligamentous thickening fuels an inflammatory response. Healthy controls were recruited in this study just before they underwent laminectomy or microdiscectomy under a general anaesthetic. They had a negative history of malignancy, inflammatory disease, autoimmune disease, asthma, epilepsy and recent illness (within a fortnight). All individuals underwent routine blood tests including full blood count (FBC), urea and electrolytes (U&E), liver function tests (LFT), coagulation screen as well as electrocardiogram (ECG), which were normal.

The preoperative concentrations of the same cytokines of patients affected by brain tumours were found to be significantly lower than the concentrations of the controls (IL-6 range between 1 pg/ml, IL-8 2.5 pg/ml and IL-10 0 pg/ml). This difference in the preoperative levels could be explained by the fact that all patients were on high dose of Dexamethasone. As this drug has anti-inflammatory effects, it is logical to think that it antagonises the inflammatory response of the patient to the tumour.

Controls on the other hand, were not administered Dexamethasone or any other steroidal drug for that matter, but most of them were taking regular analgesics in the form of paracetamol, non-steroidals and/or opioids which do not exhibit anti-inflammatory effects.

The difference in the preoperative concentrations of cytokines between controls and patients could also be explained by the drugs received by patients but not controls. For example, antiepileptic drugs (AED) such as Sodium Valproate were found to inhibit production of IL-6 [205]. These AEDs were received only by patients but not controls. Some of our patients were taking Sodium Valproate regularly, which could have influenced the concentration of the circulating IL-6.

In accordance with the previously reported literature, in this study gender did not have an effect on the preoperative cytokine levels of controls and patients alike [206]. Similarly, age did not influence the preoperative cytokine levels of the control and patient datasets. However, there is discrepancy in the literature as to whether age contributes to a change in the circulating baseline levels of the inflammatory cytokines [188, 194]. A possible explanation for the inconsistency in the various studies might be that the elderly participants were not screened for associated diseases, such as hypertension, cardiovascular disease, cerebrovascular disease, diabetes mellitus, cancer, or chronic renal disease, which could increase inflammation and hence influence the cytokine levels.

When comparing the preoperative with the postoperative concentrations of the patients all three cytokines had a significant increase. This observation can be due to the inflammatory response of the tumour as well as that of the healthy tissue undergoing the trauma of the operation. In addition, it is possible that the disruption of the blood brain barrier (BBB) during the operation might facilitate the easier entry of the cytokines in the circulating blood.

When the group of patients was subdivided into three different age groups, there was no significant difference of the pre- versus post-operative cytokine concentrations seen in the young age group (0-39 yrs). This was not the case for

the other two age groups (40-69 and 70-100 yrs). One hypothesis is that the response of the younger group to the tumour or the operation is less intense than that observed in the older age groups. As previously reported, age has a role to play in the increasing levels of the inflammatory cytokines in patients [188]. This effect was found to be exacerbated by cardiovascular or cerebrovascular disease, diabetes, genetic factors as well as environmental factors like smoking and obesity. The cytokine concentrations observed in our study might have been affected by the fact that some of our patients were suffering by one or more of the aforementioned factors. Another possibility for the lack of difference between the pre- and postoperative cytokine levels in the young age group might be its small sample size comprising only four patients. As such, the sample is potentially too small to give statistically conclusive results. On the other hand, the other two age groups were populated by a good number of individuals (16 for the 40-69 and 15 for the 70-100 years age group) making the statistical analysis more robust.

It is clear from the observed results that the operation itself plays a major role in the difference seen between the pre- and postoperative cytokine concentrations. The production and release of the inflammatory cytokines into the blood of the patients might be affected by factors like the breakdown of the BBB, the tumour neovasculature and the inflammatory response of the tumour and the healthy tissues to the operation.

It has been well established that inflammation plays an important role in the aetiopathogenesis and manifestation of pathological brain changes [207, 208]. During inflammation, activated glial cells, neurons and immune cells that invade brain tissue, produce pro-inflammatory cytokines. Within the CNS, cytokines stimulate inflammatory processes that may impair blood-brain barrier permeability, promote apoptosis of neurons and oligodendrocytes as well as induce myelin damage.

There is accumulating evidence suggesting that inflammatory cytokine levels are different between men and women [207]. This could be a result of the

hormone oestrogen and its anti-inflammatory effect within the CNS providing neuroprotection [209]. The fact that oestrogen may modulate cytokine expression, and that gender differences of cytokine production are apparent in animal models of Parkinson's disease (PD) and Multiple Sclerosis (MS), suggests an important connection between oestrogen and cytokines in neurodegeneration [207, 210]. The neuroprotective effects of oestrogens vary with reproductive age in females. Johnson and coworkers have shown that oestrogens attenuate the production of inflammatory cytokines in females. Following inflammatory stimuli, females of reproductive age generate fewer inflammatory cytokines [209].

Despite this literature evidence of a gender effect on the cytokine levels, the present study did not identify a significant difference. A possible explanation might be that the female sample size comprised mostly postmenopausal women and as such oestrogen did not affect the production of cytokines. Further recruitment of younger female patients would shed more light as to whether there is gender effect on the cytokines.

This work suggests that all three tested cytokines are suitable candidates to be used as biomarkers for malignant gliomas. Provided that the results observed are not influenced by drugs administered to the patients, these biomarkers would allow clinicians to follow up patients during the course of their disease in terms of monitoring response to treatment as well as early detection of disease recurrence. Further studies involving patients affected by malignant brain tumours who are not yet on Dexamethasone or AEDs would elucidate if the differences observed were due to the tumour or the drugs. All three cytokines are novel biomarkers for this disease as there are no previous reports on their involvement in malignant gliomas. Another novelty of this work is that there are no previous studies investigating the pre *vs*. postoperative levels of cytokines in patients with malignant gliomas. As such, this work offers a significant contribution towards a better understanding of these molecules and their role in this disease. Further studies with larger cohort of healthy controls and patients would increase the reliability of the use of these molecules as

biomarkers for malignant gliomas while it would allow novel ones to be discovered.

5 BIOMARKERS IN GLIOMAS: ANGIOGENESIS FACTORS

5.1 Introduction

The importance of biomarker discovery for malignant gliomas has been mentioned in Chapter 4 and therefore will not be discussed again here. This Chapter focuses on the use of Angiogenesis factors as potential brain tumour biomarkers.

Vasculogenesis is the *de novo* formation of blood vessels from angioblasts. These are cells that derive from the mesoderm and can differentiate into endothelial cells but have not yet acquired all the endothelial cell markers. Vasculogenesis is a complex process, involving interactions between the cells and the extracellular matrix both spatially and temporally.

Angiogenesis (sprouting and intussusceptive) is defined as the growth of new blood vessels from existing vasculature. This process is largely restricted to embryogenesis and rarely in adults, mainly during physiological menstruation and pathological wound healing. The mechanism of new vessel formation is different in the two types of angiogenesis. Sprouting angiogenesis is the formation of new vessels by sprouts of endothelial cells, which grow towards an angiogenic stimulus. It involves several steps including the enzymatic degradation of the basement membrane, the proliferation and migration of the endothelial cells (EC) towards the angiogenic stimulus, the EC tubule formation, the vessel fusion and pruning and finally the pericytes stabilisation. This type of angiogenesis adds vessels in parts of tissue that lack vasculature. Intussusceptive or splitting angiogenesis on the other hand, produces new vessel formation by splitting an existing vessel into two. This occurs when the vessel wall, extends into the lumen of the vessel, causing the single vessel to split into new vessels. This type of angiogenesis requires reorganisation of existing endothelial cells, rather than proliferation and migration. For this

reason it is thought to be faster and more efficient than sprouting angiogenesis (Figure 5-1) [211]. Pantsulaia and coworkers demonstrated that genetic, hereditary and environmental factors shared by members of the same family influence the concentrations of angiogenesis factors in a healthy population [212]. Other pathological conditions, which can stimulate angiogenesis, include atherosclerosis, osteomyelitis, diabetic retinopathy, psoriasis, and tumour growth [213].

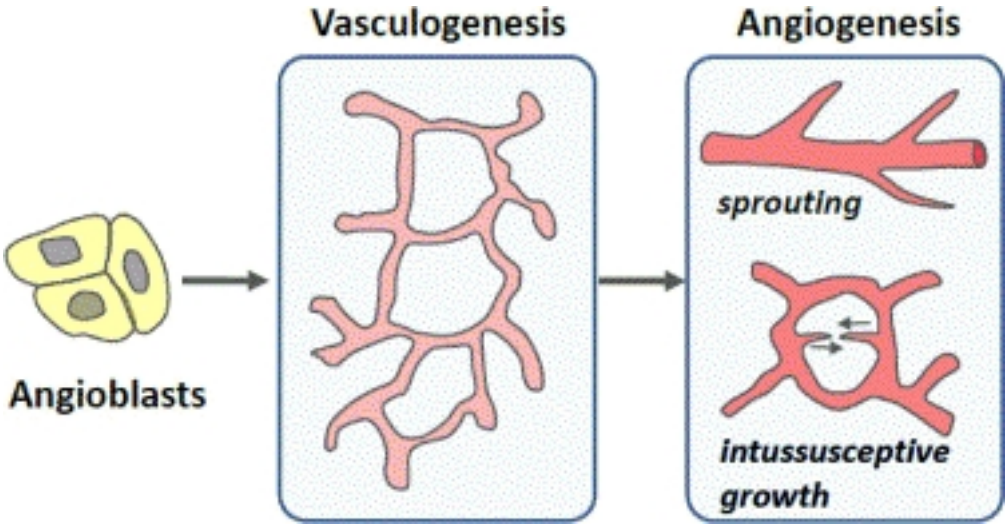

Figure 5-1 Basic types of primary vascular growth. Adapted by [211]

It is widely reported in the literature that the tumour produces angiogenesis factors in response to hypoxia, to promote its neoangiogenesis and growth [214-219].

In the case of tumour angiogenesis, the tumour cells produce angiogenesis factors that activate endothelial cells. These activated endothelial cells produce proteolytic enzymes that break down the basement membrane allowing the migration of the endothelial cells and the release of bound vascular endothelial growth factor (VEGF) into the surrounding tissue. The migrated endothelial cells start to proliferate forming tube-like structures that finally mature in new vessel formation [220].

In the last few years many already known growth factors have been identified as having angiogenic activity as well. Those include fibroblast growth factors (αFGF or FGF-1 and βFGF or FGF-2), transforming growth factors (TGF-α and TGF-β), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), tumour necrosis factor- α (TNF-α), inteleukin-8 (IL-8) and prostaglandins (PGE_1 and PGE_2) [214, 215]. Some of those factors are also considered being members of the "generic family" of cytokines.

Tumour angiogenesis is a cardinal feature for tumour growth as well as for the development of metastatic disease. Several pro-inflammatory cytokines, such as IL-1, IL-6, and TNF, stimulate VEGF gene expression in a tissue-specific way. It is known that VEGF mRNA is produced in five isoforms by nonmalignant as well as malignant cells. Hypoxia, hypoglycemia and inflammation are the triggers for nonmalignant production of VEGF.

There is a fine balance between activating and inhibiting mediators controlling angiogenesis in tumours [214]. Malignant cells produce VEGF constitutively in high concentrations even in normoxic conditions, due to genetic alterations. VEGF is a protein that binds the basal membrane of the endothelium initiating several effects on the endothelial cells, from stimulation of mitosis, to inhibiting apoptosis and increasing permeability to the vascular endothelium. Its role is important both in physiological as well as pathophysiological angiogenesis (embryogenesis, physiological menstrual cycle, abnormal wound healing) [221]. Several previous studies have investigated the site of production of VEGF. Several organs such as lungs, adrenal glands, liver, heart, stomach mucosa and kidneys have been found to produce this protein. It is also known that platelets and leukocytes release VEGF during blood clotting. Today, it is assumed that all tissues have the potential to produce this growth factor [222].

Normal physiological concentrations of VEGF have been measured by several studies. It is clear when looking at the literature that the reported "normal ranges" are in many studies different by two to three hundred-fold between

studies. This is mainly due to the fact that the assays used measure total free circulating VEGF (competitive immunoassays) as others measure only free VEGF (immunoassays with neutralizing antibodies). If the antibodies of the immunoassay are specific to one of the several isoforms of VEGF, the results are different again. Studies using competitive ELISAs reported concentrations of total free VEGF between 3-25 $x10^9$ pg/mL, a value that drops to 1 $x10^9$ pg/mL when Radioimmunoassays (RIAs) are used [223-225]. When the different isoforms are measured the concentrations reported change again. Free $VEGF_{121}$ has been reported as 19 pg/mL [226] whereas free $VEGF_{165}$ was 42 pg/mL [227]. The most commonly used commercial ELISA (Quantikine) detects both free isoforms $VEGF_{121}$ and $VEGF_{165}$. Studies using this assay report plasma values of 9–150 pg/mL [227-231].

Jelkmann reports higher values when an in-house assay with polyclonal antibodies to detect VEGF was used [232]. Although these values are independent of gender, interference with molecules that bound VEGF (i.e. heparin, 2-macroglobulin), as well as the type of sample used (serum versus plasma), are factors that are thought to play a crucial role into influencing the results of the assay used. When serum is used as sample type to measure VEGF, the reference interval for $VEGF_{121/165}$ is relatively high, averaging 10–300 pg/mL [227, 233-237].

This difference in measured concentrations between the two samples (serum v plasma) is due to the VEGF released from platelets and leukocytes during clotting. It is thought that in fact, serum VEGF concentrations reflect blood platelet counts rather than VEGF produced by peripheral tissues [229, 235, 238]. It has been reported that not only clotting, but also its duration as well as temperature increases serum VEGF concentration [236, 238, 239]. When free $VEGF_{121/\ 165}$ is measured in blood cells and plasma the values reported are 445 pg/mL and 19 pg/mL respectively.

Jelkmann goes as far as recommending that citrated, EDTA-treated, or heparinized plasma processed in glass tubes, frozen to -80^0 within one hour of

venopuncture, should be the material of choice for measurement of VEGF [235]. Wynendaele and coworkers suggest that in order to stabilize platelets the blood should be collected in CTAD tubes, which contain citrate, theophylline, adenosine, and dipyridamole [240]. Several studies have shown that the concentration of circulating free $VEGF_{121/165}$ is elevated in malignant disease, [240, 241] and that it increases further during the development of metastasis (breast [242], gastrointestinal [243], colorectal [230], prostate cancer [244] and melanoma [231]). Kondo and coworkers were the first to recognize VEGF as a potential serum biomarker in malignant disease [245]. Several cancers such as brain, lung, gastrointestinal, hepatobiliary, renal, ovarian and others, are known to have increased serum free VEGF levels. Although platelets are the main culprits for the serum concentrations of VEGF as previously mentioned, Lee and coworkers demonstrated that blood platelets in tumor patients contain more releasable VEGF than platelets from healthy persons [246]. They also observed that tumour cells have been forming aggregates with platelets in metastasis. By measuring VEGF in both healthy and cancer patients in serum and plasma, they demonstrated that VEGF in serum is significantly higher than matched plasma as well as that the VEGF difference between the two sample types correlated with the platelet count of both healthy and cancer affected individuals. They argue that platelet-derived VEGF reflects the biology of cancer cells, and as such serum is more useful than plasma in the diagnosis and follow-up of malignancies [246].

However, it is almost impossible to carry out interlaboratory comparisons of VEGF serum data, mainly because the procedures for blood handling are not standardized with respect to clotting material, duration, and temperature. Therefore, Banks and coworkers suggest that plasma should be the sample type of choice for VEGF measurement, in order to make more accurate and most importantly comparable results between studies [235].

It is known that platelets secrete VEGF, platelet-derived growth factor (PDGF), platelet factor 4 (PF4), thrombospondin-1 (TSP-1), fibroblast growth factor, basic (bFGF), and endostatin. Peterson and coworkers reported as normal

ranges of the above mentioned growth factors in platelets the following: VEGF (0.74 ± 0.37 pg/106 platelets); PDGF (23 ± 6 pg/106); PF4 (12 ± 5 ng/106); TSP-1 (31 ± 12 ng/106); bFGF (0.44 ± 0.15 pg/106); and endostatin (5.6 ± 3.0 pg/106). They showed that there was an excellent correlation between the platelet levels calculated with the actin ELISA and complete blood count. They claim that the measurements are accurate and reproducible with very little biovariability, suggesting that platelet-derived measurements for the angiogenesis factors should be the gold standard rather than plasma or serum [247]. Age as well as gender does not influence VEGF levels [248, 249].

Platelet derived growth factor (PDGF) is another growth factor that plays an important role in cell proliferation, migration, wound healing and angiogenesis. It stimulates mitosis as well as differentiation for cells of mesenchymal origin such as smooth muscle cells, glial cells and fibroblasts. It regulates proliferation both in physiological and pathological conditions. There are several cells that are found to produce PDGF. Amongst others, platelets, fibroblasts, endothelial cells, pericytes and glioma cells.

In the CNS, PDGF is secreted both during embryogenesis as well as in the adult [250]. It has been found in neurons, pericytes and astrocytes. As well as stimulating cell division, proliferation and differentiation of progenitor neural cells during embryogenesis [251] and in the adult brain [252] it is also neuroprotective during injury [253-255]. It is well known that amplification of PDGF and PDGFR genes is frequent in human gliomas of all grades [256]. Its mode of expression suggests that it has an autocrine and paracrine effect on the tumour cells [257].

There are several studies that report the physiological concentrations of PDGF. The serum concentration reported varies slightly between studies from 1700pg/ml [258] to 1985 (±1028) pg/ml [259]. Plasma levels of PDGF have also been reported and as for VEGF, these have also a great range of variability between studies. The concentrations were ranging from not detectable (below

1.56 fmol/100 microliters) [260] to 450 (±140) pg/ml [261]. Age as well as gender does not influence PDGF-BB concentrations [262]

Follistatin (FLT) is a protein that is expressed in nearly all tissues. Its main function is to bind and neutralise TGF. It has been reported that FLT suppresses invasion in several malignancies (lung, ovarian and endometrial cancer, as well as oral squamous carcinoma)[263-266]. Several studies in the literature report the "normal range" of FLT, but as with VEGF and PDGF, its concentrations vary with the different assays used as well as the type of sample used to make the measurement.

Widera and coworkers reported that serum concentrations of FLT are higher than that observed in citrated plasma and heparin-treated plasma by approximately 18%. The median concentration of apparently healthy individuals in serum is 7180 (range 1060-18490) pg/mL [267].

Wakatsuki and coworkers reported as normal human serum FLT levels 13300 (± 4700) pg/mL. They observed that FLT is present during puberty and the concentrations tend to increase with age and during pregnancy [268, 269]. Neither gender, nor the stage of the menstrual cycle had an effect on the concentration of circulating FLT (approximately 450 pg/ml). Interestingly, postmenopausal women had higher FLT concentrations than premenopausal women (approximately 1400 pg/ml) [270].

Sakamoto and coworkers reported that subjects with chronic kidney disease, chronic liver disease, advanced solid cancer as well as haematological malignancies have higher concentrations of FLT than apparently healthy individuals [271].

5.2 Aims

This study explores the feasibility of collecting serum and plasma samples from patients and healthy volunteers (controls) pre- and post-operatively. In addition it investigates the use of angiogenesis factors VEGF, PDGF-BB and Follistatin as potential biomarkers for malignant brain tumours. Furthermore, it scrutinises the effect of parameters such as the presence of brain tumour, the operative stage, the age and the gender to the serum levels of these angiogenesis factors.

5.3 Materials and methods

Serum samples were collected from 36 patients and 36 controls immediately pre-operatively and within 24 hours after the operation. Control patients were defined as patients treated surgically under a general anaesthetic in Preston's Neurosurgical Unit for benign disease (degenerative spinal disease such as disc protrusion or ligamentous thickening), with no past medical history including no known malignancy. None of the controls were on regular medications and in particular steroids of any form. The concentrations of 8 angiogenesis factors were measured using the Bio-Plex Pro Angiogenesis Assay (Bio-Rad) according to manufacturer's instructions [201]. The protocol is briefly outlined below.

Multiplex bead-based immunoassay

Simultaneous measurement of serum concentrations of Angiopoietin, Follistatin, Granulocyte-colony stimulating factor (G-CSF), Hepatocyte growth factor (HGF), IL-8, Leptin, Platelet-derived growth factor (PDGF-BB), Platelet/Endothelial cell adhesion molecule-1 (PECAM-1) and Vascular endothelial growth factor (VEGF), was performed using commercially available multiplex bead-based sandwich immunoassay kits (Bioplex) as per the manufacturer's instructions. Briefly, serum samples (50 µl per well) or standards (50 µl per well) were incubated with 50 µl of the pre-mixed bead sets in pre-wetted 96- well micro titer plates at room temperature for 30 minutes. After washing, 25 µl of the fluorescent detection antibody mixture was added for 30 min. After washing further 50 µl of streptavidin-phycoerythrin was added to each well for an additional 10 min at room temperature. After washing 125 microliters of assay buffer was added to each well. The plate was shaken at 1100rpm for 30 sec at room temperature prior to placing it into the reader. A range of 3.2-10,000 pg/mL recombinant full-length angiogenesis proteins was used to establish standard curves and to maximize the sensitivity and dynamic range of the assay. Angiogenesis levels were determined using a Bio-Plex 200

System (Bio-Rad Laboratories INC), and the Bio-Plex Manager software was used for data acquisition and analysis. The data are reported as relative median fluorescent intensities [196].

5.4 Results

5.4.1 Biomarker analysis

Eight biomarkers measured using the Bio-Plex Pro Angiogenesis and Cytokines Assay (Bio-Rad). Of these, three angiogenesis factors (VEGF, PDGF-BB and Follistatin) were the most significant ones. The raw measurements from these three biomarkers are summarized in Appendix 4.

Outlier removal

Outliers were removed using the Fourth-Spread method as described in Chapter 4. Once the outliers were removed, the remaining data presented a more uniform spread as shown in the three boxplots (Figure 5-2, Figure 5-3 Figure 5-4).

Checking datasets for normality using Shapiro-Wilk Normality test

Before deciding which statistical method to be used for the data analysis, the data where checked whether they fit a normal distribution using the Shapiro-Wilk Normality test [203]. The null hypothesis for the Shapiro-Wilk test is that the data are normally distributed. If the chosen cut off score is 0.05 and the p-value is less than 0.05, then the null hypothesis that the data are normally distributed is rejected. If the p-value is greater than 0.05, then the null hypothesis has not been rejected and therefore the data are normally distributed.

According to this test almost all measurements derived from the Angiogenesis factors did resemble a normal distribution and thus the Student's paired t-test was used [272]. The results from the Shapiro-Wilk normality test are

summarised in Table 5-1, Figure 5-2 *Boxplot summarising the control (pre- only) and the pre- and post-operative concentrations (pg/ml) of VEGF measured in the malignant patients.*

Table 5-1 ***The Shapiro-Wilk scores and p-values as determined for the various datasets.***

Shapiro-Wilk Normality Test	
Angiogenesis factor data	
VEGF-Pre	W = 9.66E-01, p-value = 0.422
VEGF-Post	W = 9.54E-01, p-value = 0.247
PDGF-BB-Pre	W = 9.66E-01, p-value = 0.464
PDGF-BB-Post	W = 9.47E-01, p-value = 0.150
Follistatin-Pre	W = 9.81E-01, p-value = 0.852
Follistatin-Post	W = 9.24E-01, p-value = **<0.05**
Control-VEGF	W = 9.05E-01, p-value = **<0.05**
Control-PDGF-BB	W = 9.91E-01, p-value = 0.999
Control-Follistatin	W = 9.54E-01, p-value = 0.359

Values above the 0.05 cut off score suggest normal distribution of the respective datasets. Generally the Angiogenesis factors datasets appear to be distributed normally. Significant values are shown in bold.

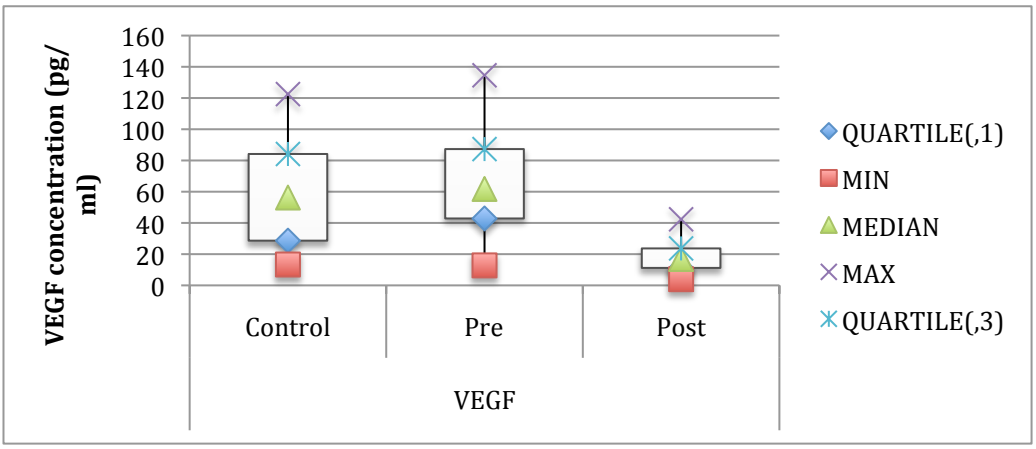

Figure 5-2 *Boxplot summarising the control (pre- only) and the pre- and post-operative concentrations (pg/ml) of VEGF measured in the malignant patients.*

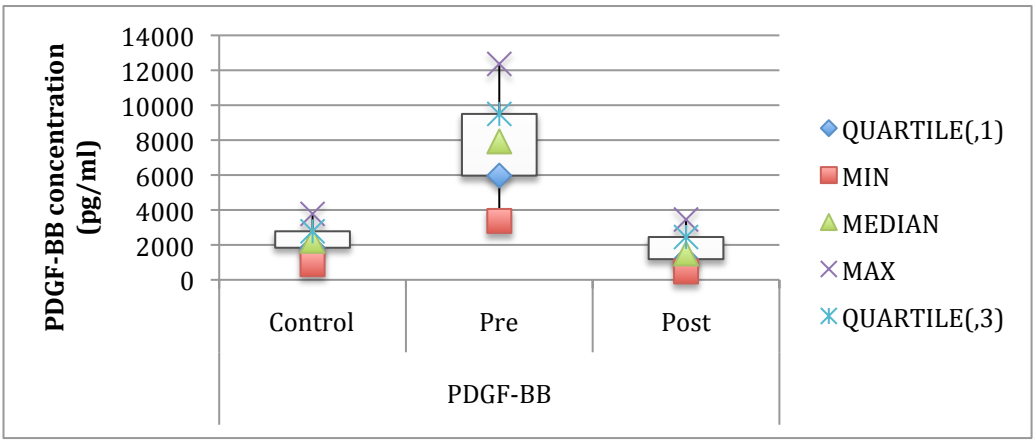

Figure 5-3 *Boxplot summarising the control (pre- only) and the pre- and post-operative concentrations (pg/ml) of PDGF-BB measured in the malignant patients.*

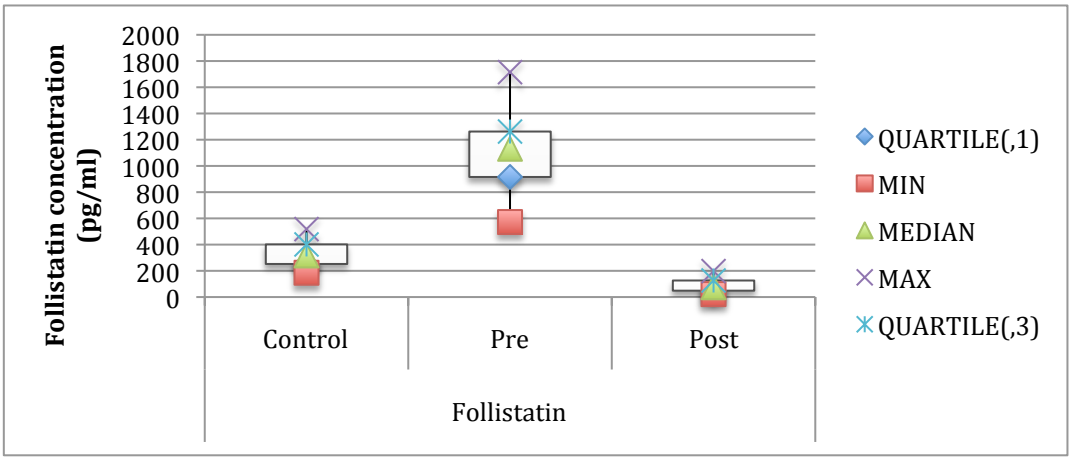

Figure 5-4 *Boxplot summarising the control (pre- only) and the pre- and post-operative concentrations (pg/ml) of Follistatin measured in the malignant patients.*

In terms of evaluating statistical significance given a calculated p-value, both significance levels $\alpha=0.001$ (conservative) and $\alpha=0.05$ (widely used) were considered. If a test of significance gives a p-value lower than the significance level α, the null hypothesis is rejected and the results are considered as 'statistically significant'.

Statistical package

The statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.15.1) running under Windows Vista OS with 2GB RAM [148]. All the statistical tests used here were included in the R Stats package.

5.4.2 Angiogenesis Factors analysis

The results of the analysis of the angiogenesis factors VEGF, PDGF-BB and Follistatin are summarised in the table below (Table 5-2). A more detailed description of the results is provided in the sections below.

Table 5-2 ***Summary of the p-values as resulted from the statistical analysis of the angiogenesis factors (VEGF, PDGF-BB and Follistatin) across the different datasets. Significant values are shown in bold.***

	Age group	Angiogenesis factors		
		VEGF	PDGF-BB	Follistatin
Controls *vs.* Pre-op Patients	0-100	0.472	**<0.001**	**<0.001**
Controls *vs.* Pre-op Patients	0-39	0.655	0.072	**<0.001**
Controls *vs.* Pre-op Patients	40-69	0.928	**<0.001**	**<0.001**
Controls *vs.* Pre-op Patients	70-100	0.130	**<0.001**	**<0.001**
Pre-op *vs.* Post-op Patients	0-100	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op Patients	0-39	0.398	0.057	**<0.001**
Pre-op *vs.* Post-op Patients	40-69	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op Patients	70-100	**<0.001**	**<0.001**	**<0.001**

Pre-operatively angiogenesis factors level comparison between Controls and Patients (all age groups)

To determine whether the levels of three angiogenesis factors (VEGF, PDGF-BB, and Follistatin) on patients with malignant tumour were different from the levels in patients with benign diseases and no malignancy, the datasets were compared using the Student's paired t-test. The results of the test are shown on the first row of Table 5-2 and Figure 5-6 and Figure 5-7.

The levels of two of the three Cytokines were significantly different (PDGF-BB $P<0.001$; Follistatin $P<0.001$) between patients with malignant tumour and patients with benign disease prior to the operation. VEGF didn't exhibit any significant difference between the controls and the patients prior to the operation.

These results were further analysed in order to establish whether this trend was common across our arbitrary assigned age groups: 0-39, 40-69, 70-100 of years of age.

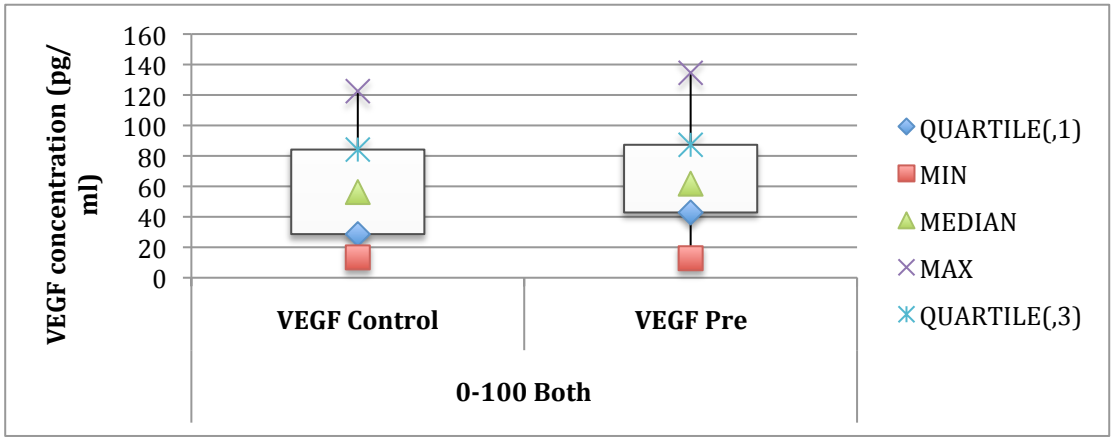

Figure 5-5 Boxplot summarizing the pre-operative serum concentration (pg/ml) of VEGF measured in the malignant patients and the controls (pre- only) across all age groups.

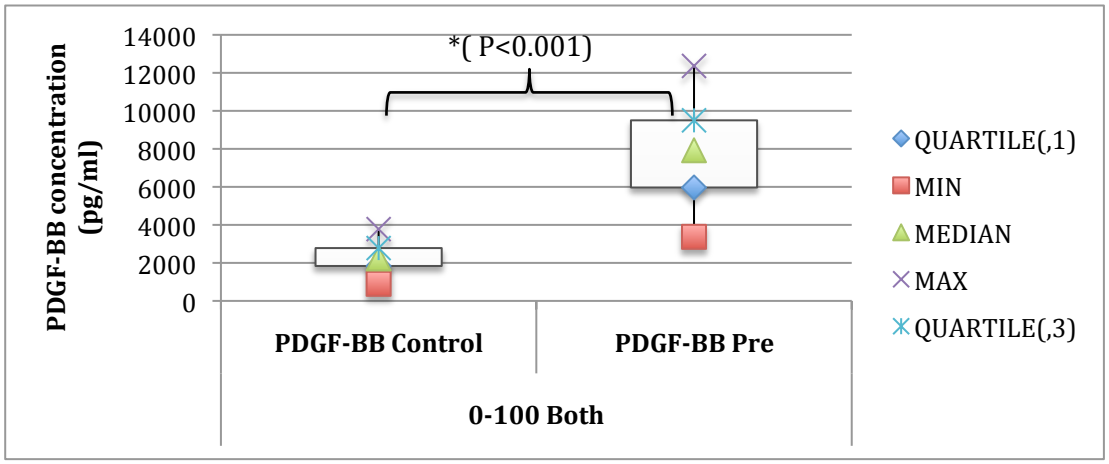

Figure 5-6 Boxplot summarizing the pre-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients and the controls (pre- only) across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.

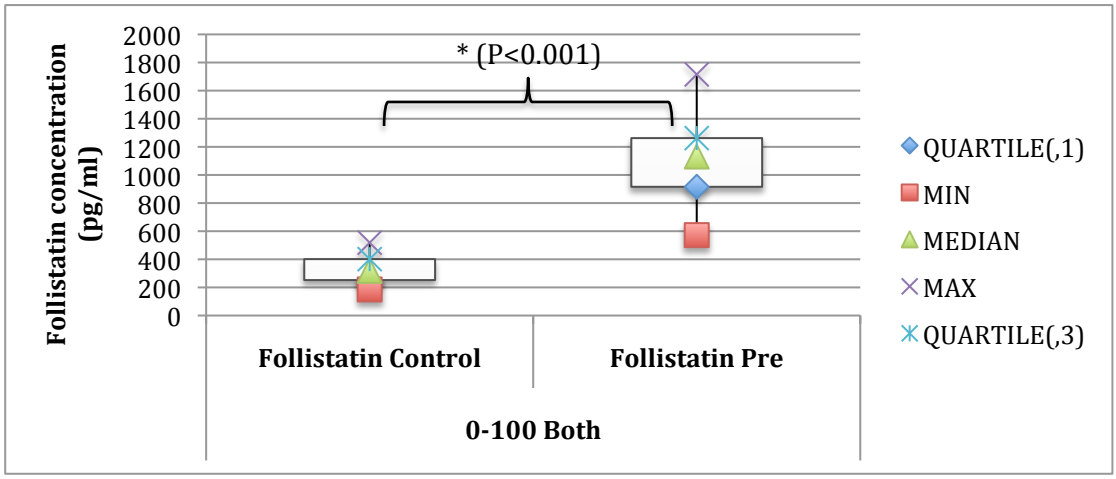

Figure 5-7 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients and the controls (pre- only) across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre-operative angiogenesis factors level comparison between Controls and Patients (age group 0-39 years)

The pre-operative levels of the angiogenesis factors assayed were tested in order to identify any statistical difference between the Controls and the tumour patients pre-operatively for the age group 0-39 years. The results from the Student's paired t-test for the 0-39 years age group are shown on the second row of Table 5-2 and Figure 5-8, Figure 5-9 and Figure 5-10.

Amongst the three angiogenesis factors assayed, only Follistatin showed difference on its levels between the Controls and the tumour patients pre-operatively for this age group ($P<0.001$).

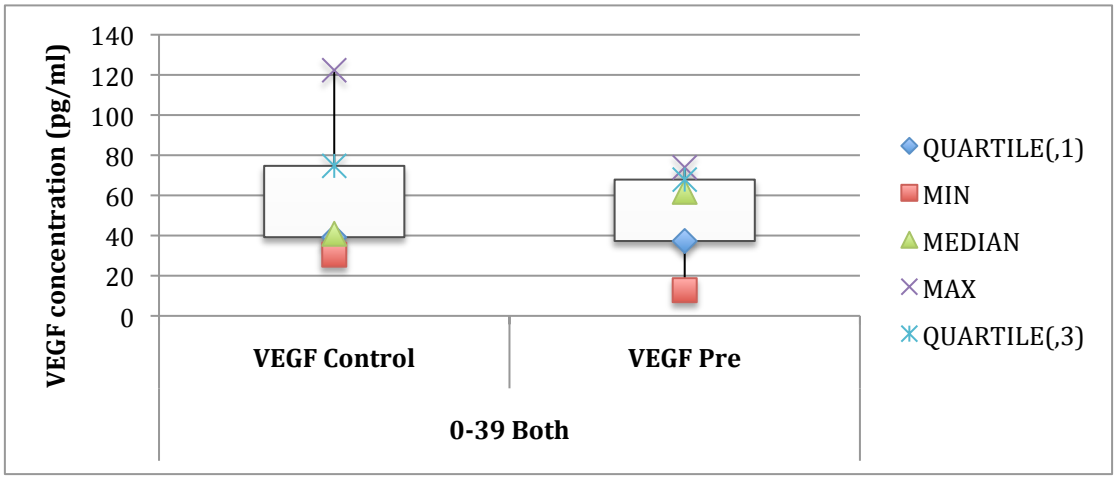

Figure 5-8 Boxplot summarizing the pre-operative serum concentration (pg/ml) of VEGF measured in the malignant patients and the controls (pre- only) across the 0-39 age group.

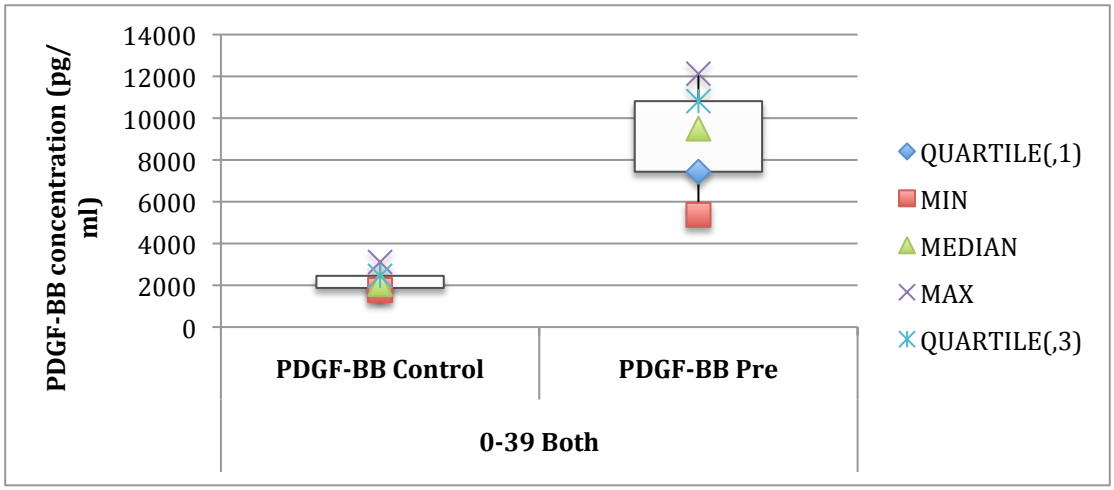

Figure 5-9 Boxplot summarizing the pre-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients and the controls (pre- only) across the 0-39 age group.

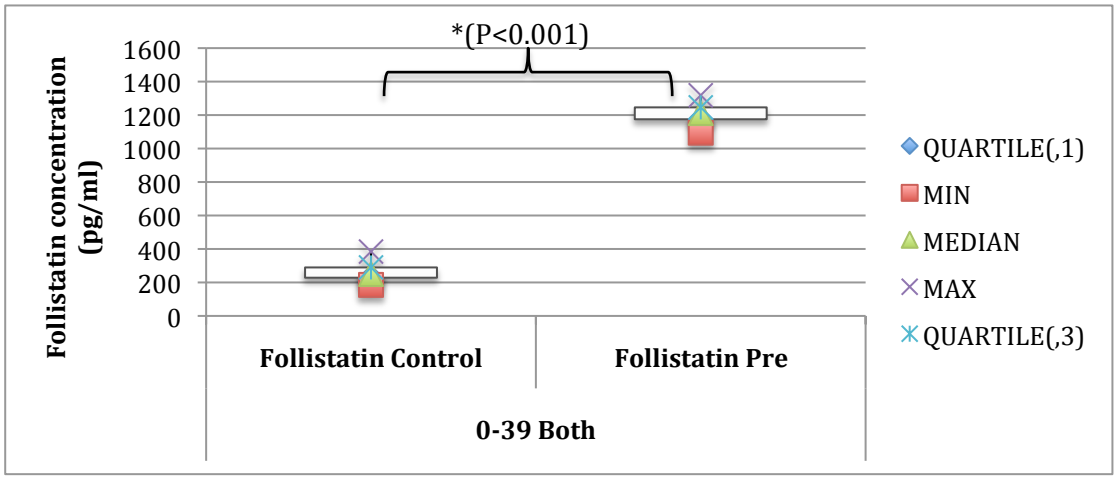

Figure 5-10 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients and the controls (pre- only) across the 0-39 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre-operative angiogenesis factors level comparison between Controls and Patients (age group 40-69 years)

Similarly, the levels of pre-operative angiogenesis factors between the Controls and the tumour patients for the age group 40-69 years were compared. The results from the Student's paired t-test for this age group are shown on the third row of Table 5-2 and Figure 5-11, Figure 5-12 and Figure 5-13.

Based on these results, the levels of both PDGF-BB and Follistatin in this age group were significantly different between the Controls and the tumour patients (PDGF-BB $P<0.001$; Follistatin $P<0.001$).

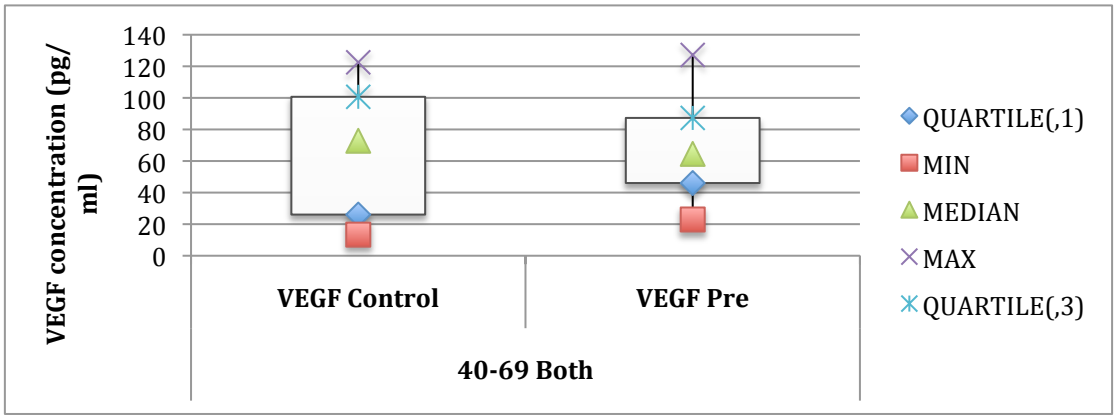

Figure 5-11 Boxplot summarizing the pre-operative serum concentration (pg/ml) of VEGF measured in the malignant patients and the controls (pre- only) across the 40-69 age group.

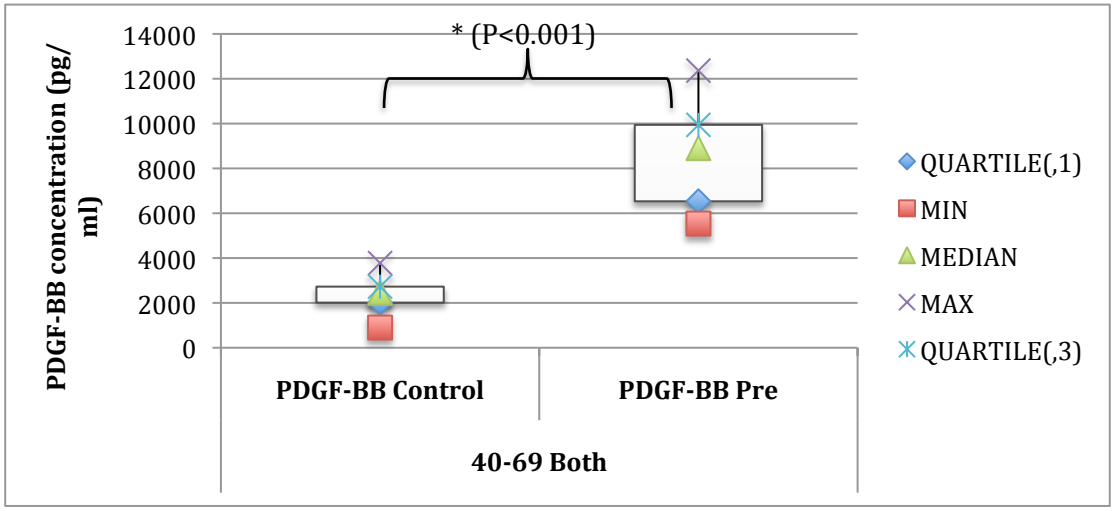

Figure 5-12 Boxplot summarizing the pre-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients and the controls (pre- only) across the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.

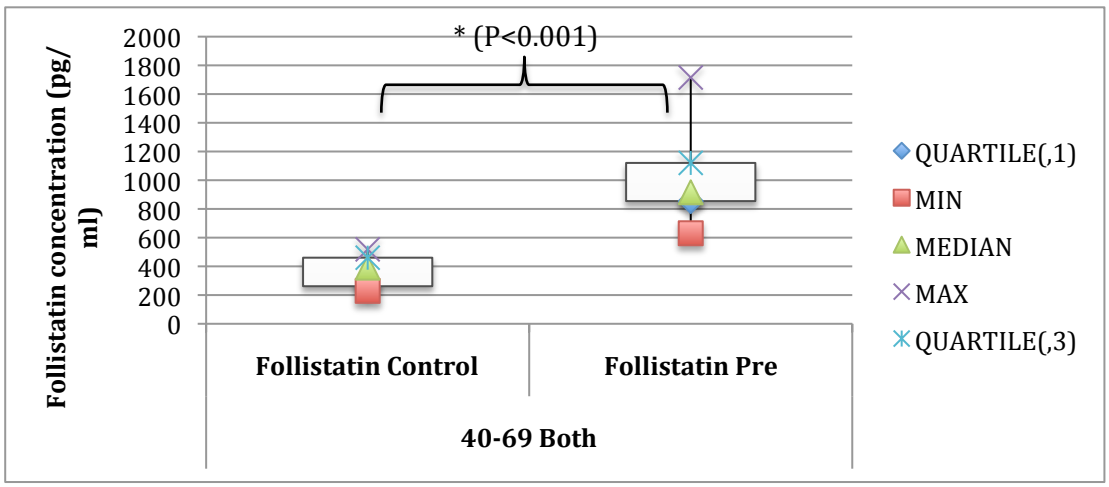

Figure 5-13 Boxplot summarizing the pre-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients and the controls (pre- only) across the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.

Pre-operative angiogenesis factors level comparison between Controls and Patients (age group 70-100 years)

The last age group to be tested for any difference in the pre-operative angiogenesis factor levels between the Controls and the tumour patients was the 70-100 years. The results from the Student's paired t-test are shown on the fourth row of Table 5-2 and Figure 5-14, Figure 5-15 and Figure 5-16.

The analysis has shown that both PDGF-BB and Follistatin levels showed difference for this age group between the Controls and the tumour patients pre-operatively ($P<0.001$).

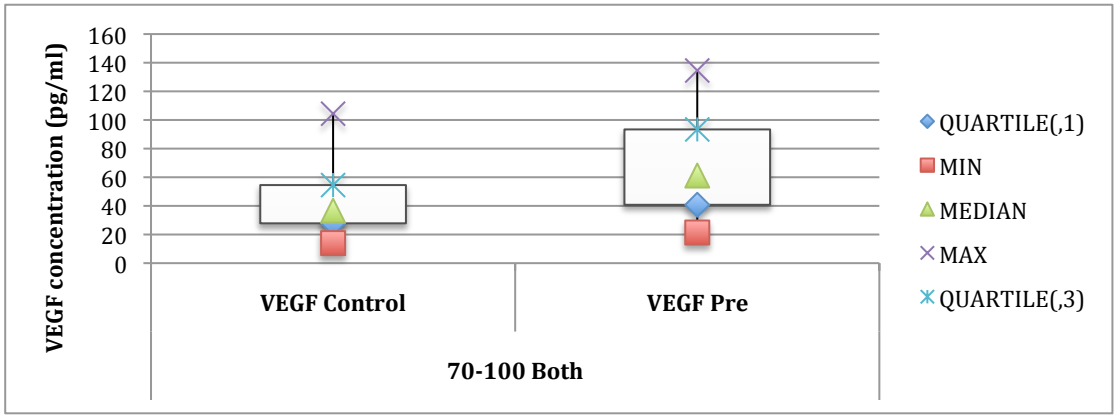

Figure 5-14 Boxplot summarizing the pre-operative serum concentration (pg/ml) of VEGF measured in the malignant patients and the controls (pre- only) across the 70-100 age group.

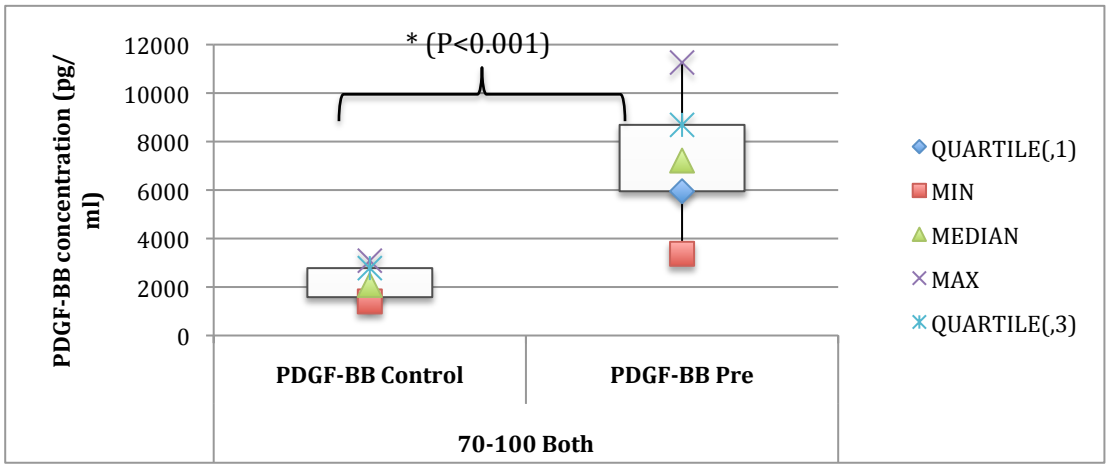

Figure 5-15 Boxplot summarizing the pre-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients and the controls (pre- only) across the 70-100 age group. ***Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

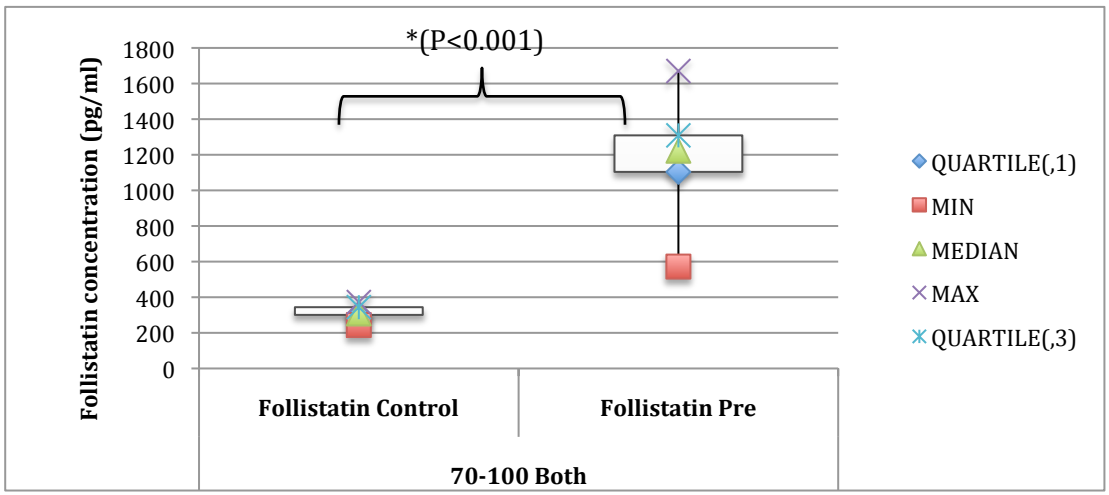

Figure 5-16 ***Boxplot summarizing the pre-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients and the controls (pre- only) across the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.***

Comparison of pre- vs. post-operation levels of Angiogenesis factors (all age groups)

Similar to the cytokines study, this study tries to answer whether there is a (statistically) significant difference in the levels of patient's angiogenesis factors pre- and post-operatively. To address this question, the Student's paired t-test was applied on the measured angiogenesis factors concentrations and the results from the Student's paired t-test are shown on the fifth row of Table 5-2 and Figure 5-17, Figure 5-18 and Figure 5-19.

The levels of all three angiogenesis factors were significantly different ($P<0.001$) on patients prior to and after the operation.

Like with the cytokines study, since there was indeed a strong difference on the angiogenesis factors levels pre- and post-operatively, the data were further analysed in order to check if this trend was common on all three age groups: 0-39, 40-69, 70-100 years.

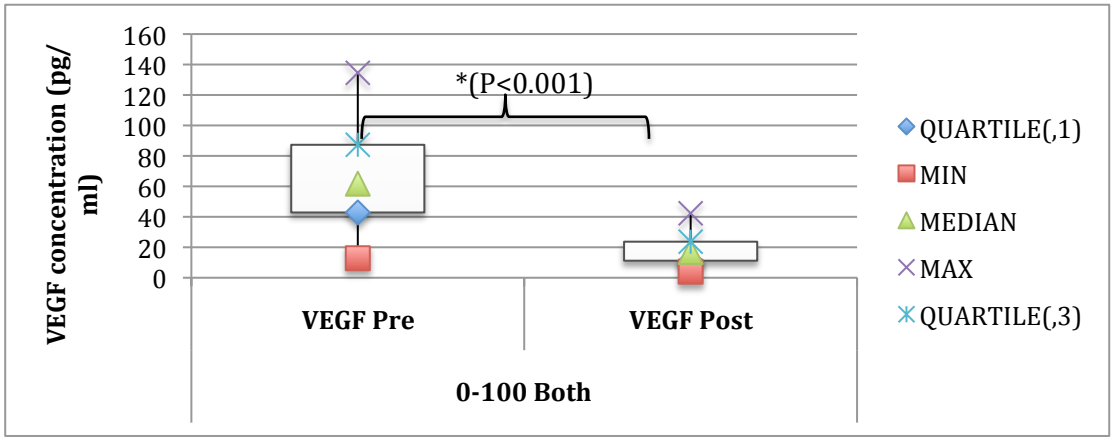

Figure 5-17 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

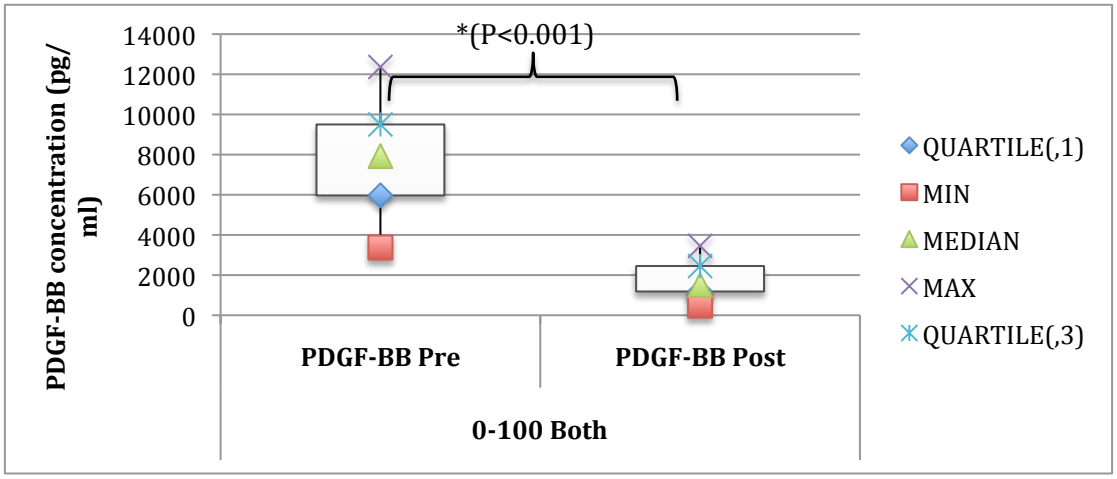

Figure 5-18 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

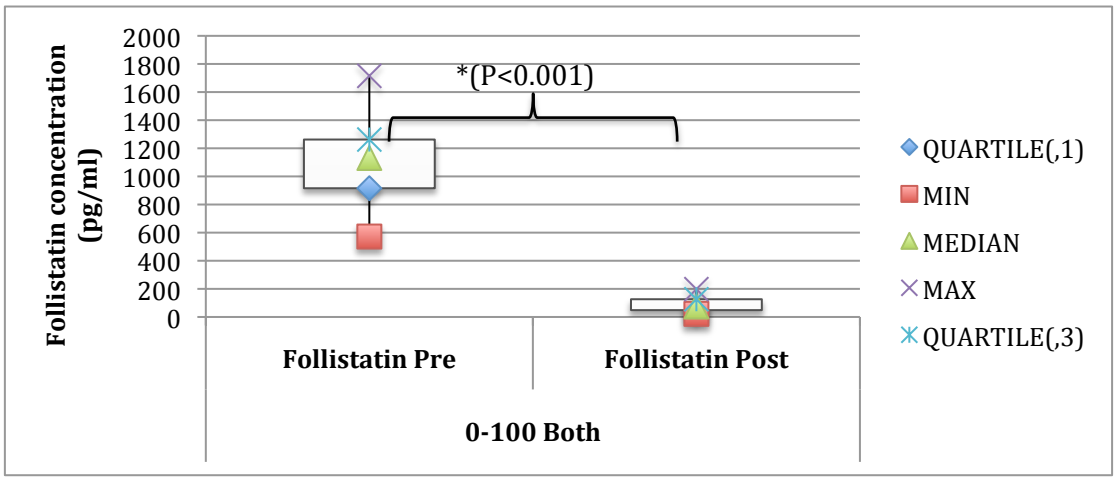

Figure 5-19 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre- vs. post-operative angiogenesis factors level comparison (age group 0-39 years)

The levels of the pre and post-operative angiogenesis factors were analysed further in order to identify a statistical difference between the samples for the age group 0-39 years. The results from the Student's paired t-test for the 0-39 years age group are shown on the sixth row of Table 5-2 and Figure 5-20, Figure 5-21 and Figure 5-22.

Amongst the three angiogenesis factors tested, only Follistatin showed difference on its levels pre- and post- operatively ($P<0.001$) for this age group whereas PDGF-BB was just above the 5% cut off value (0.05684).

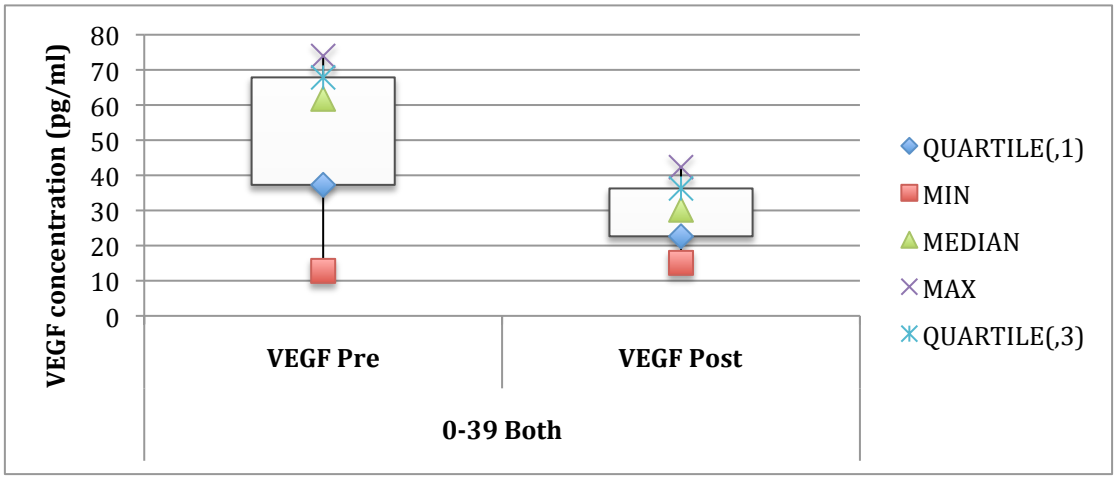

Figure 5-20 Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the malignant patients across the 0-39 age group.

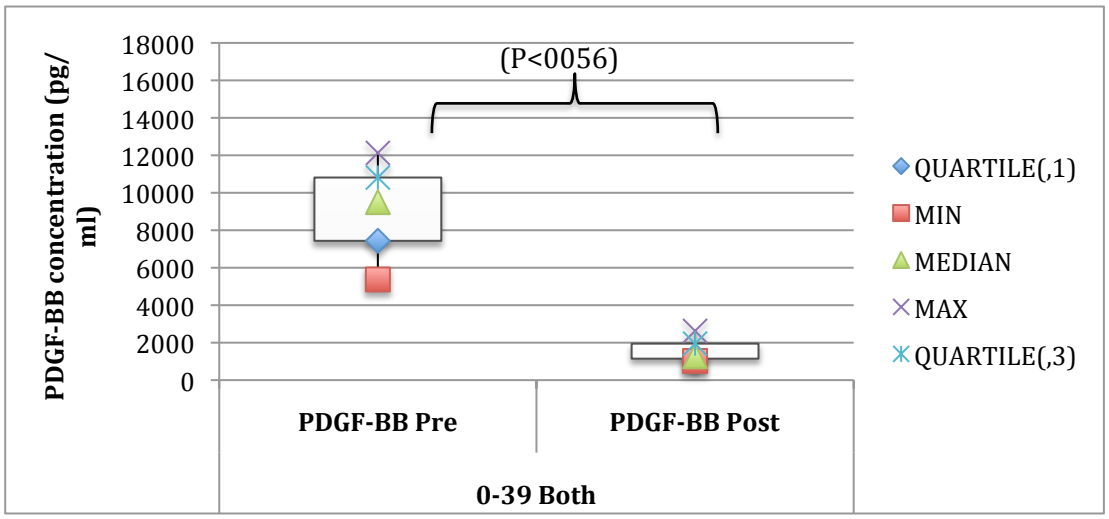

Figure 5-21 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients across the 0-39 age group. P value shown in parenthesis.*

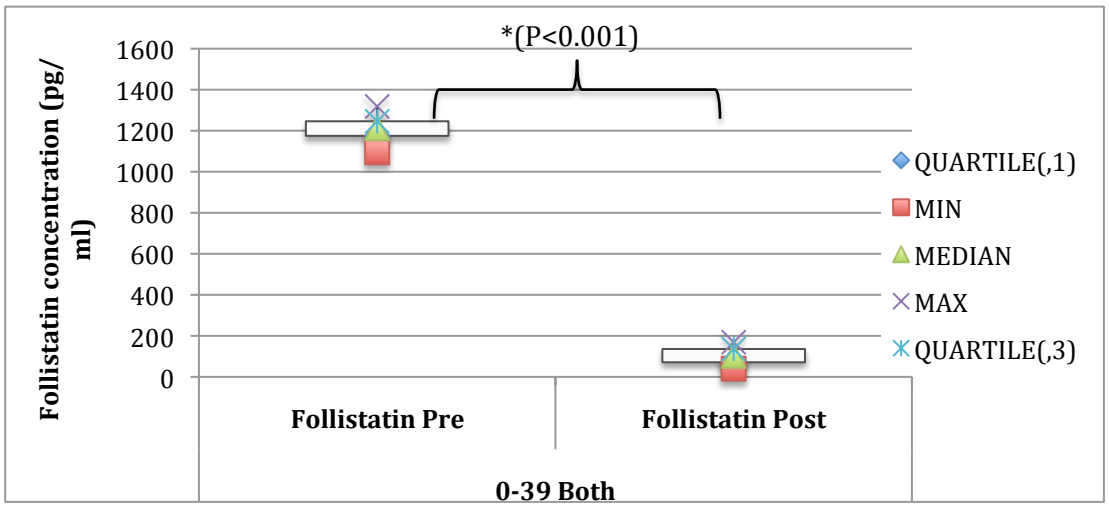

Figure 5-22 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients across the 0-39 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre- vs. post-operative angiogenesis factors level comparison (age group 40-69 years)

Similarly, the levels of angiogenesis factors between the pre- and post-operative samples for the age group 40-69 years were analysed to check for statistical difference. The results from the Student's paired t-test for this age group are shown on the seventh row of Table 5-2 and Figure 5-23, Figure 5-24 and Figure 5-25.

Based on these results, the levels of all three angiogenesis factors in this age group were significantly different ($P<0.001$) on patients prior to and after the operation.

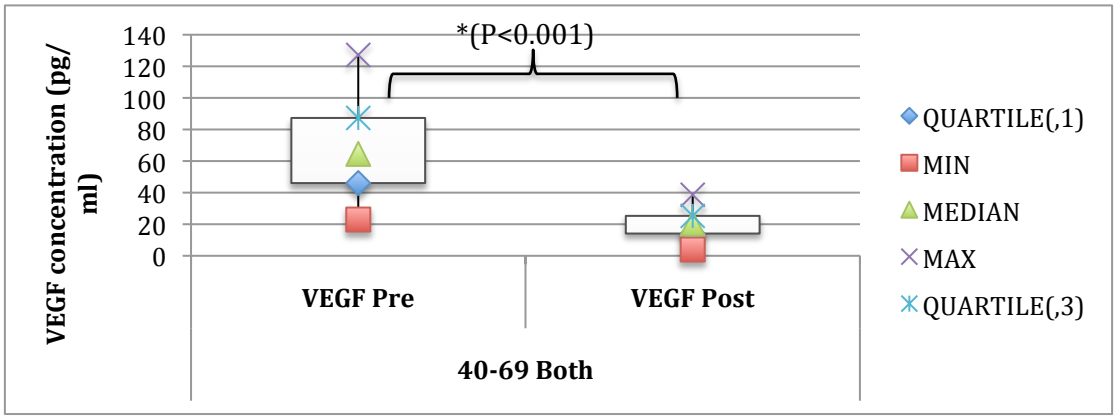

Figure 5-23 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the malignant patients across the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

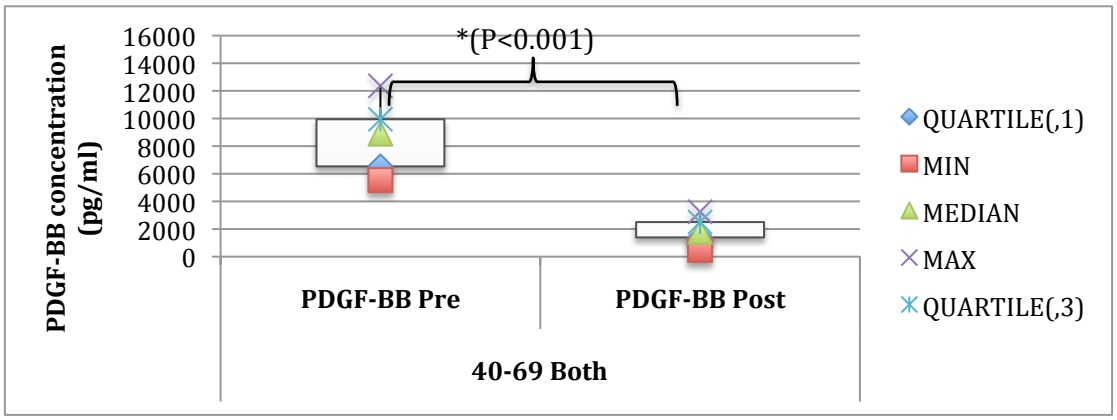

Figure 5-24 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients across the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

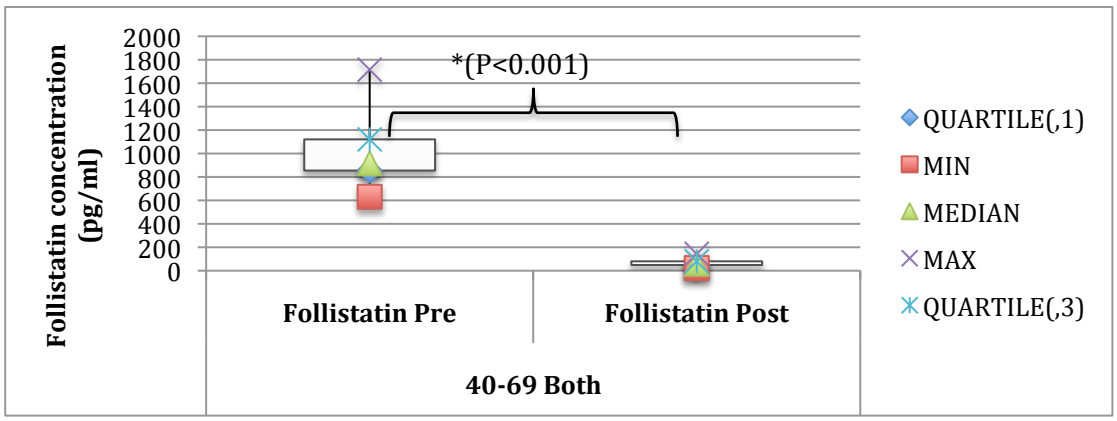

Figure 5-25 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients across the 40-69 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Pre- vs. post-operative angiogenesis factors level comparison (age group 70-100 years)

The last age group to be tested for any difference in the angiogenesis factor levels pre- and post operatively was the 70-100 years. The results from the Student's paired t-test are shown on the eighth row of Table 5-2 and Figure 5-26, Figure 5-27 and Figure 5-28.

The analysis has shown that the levels of all three angiogenesis factors in this age group were significantly different ($P<0.001$) on patients prior to and after the operation.

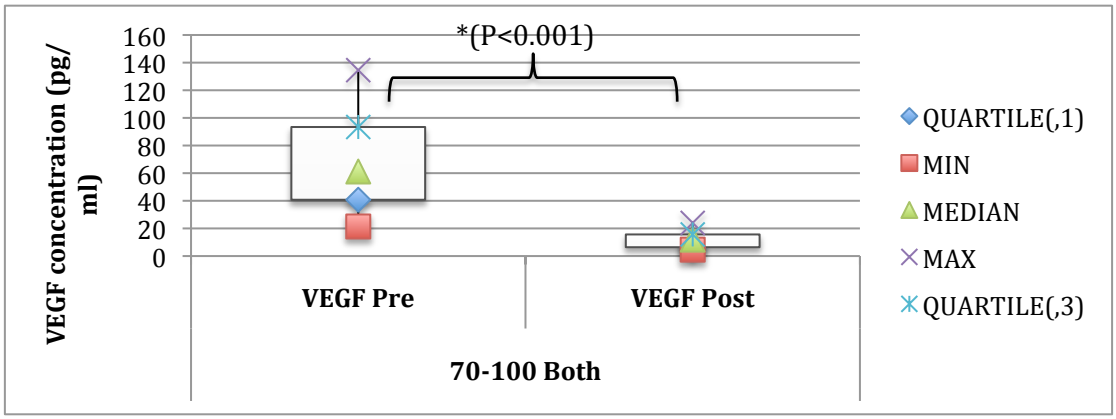

Figure 5-26 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the malignant patients across the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

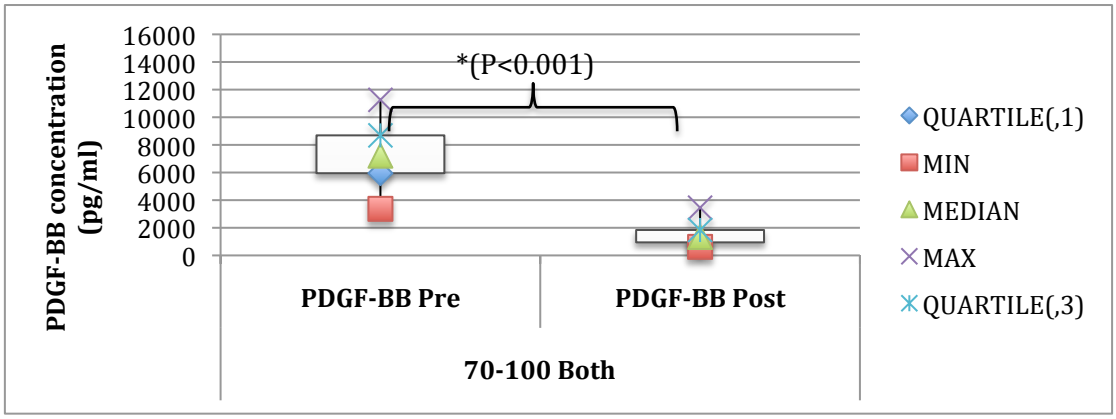

Figure 5-27 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the malignant patients across the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

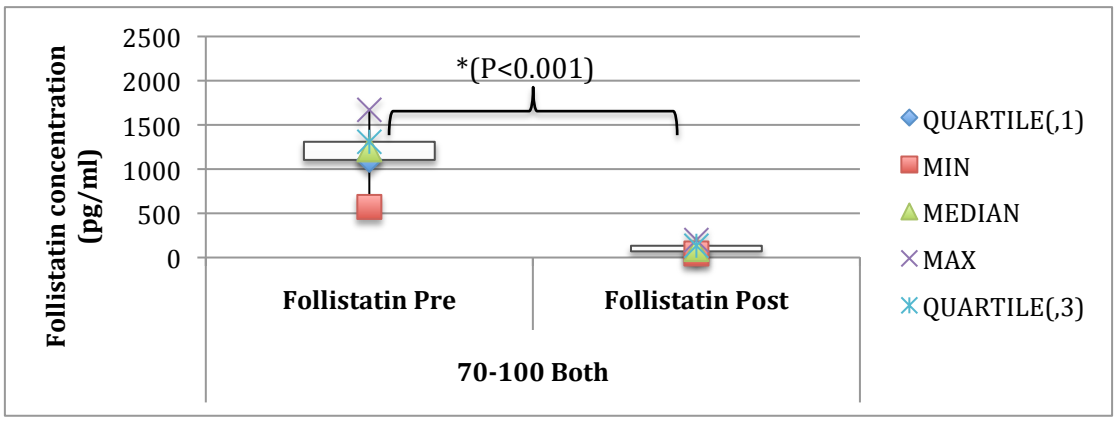

Figure 5-28 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the malignant patients across the 70-100 age group. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

Age group comparison within the pre- and the post-operation datasets

In the above analysis it has been established whether there was any difference in the levels of angiogenesis factors tested between the pre- and post-operative samples and between the Controls and the pre-operation patients for the different age groups.

The next thing to look into is whether age plays a role on the levels of the angiogenesis factors pre- and post-operatively as well as in the Controls. Using the Student's paired t-test the levels of angiogenesis factors were compared between the three age groups in the pre- or post-operatively datasets. The results are shown in Table 5-3, Table 5-4, Table 5-5 and Table 5-6 respectively.

Overall no significant difference on the levels of the tested angiogenesis factors was observed between the different age groups for either the Controls, or the patients prior to or after the operation (α=0.05). The only exception was the comparison between the age groups 40-69 years and 70-100 years post-operatively as the two age groups appeared to have significantly different levels for both VEGF and Follistatin ($P<0.05$).

Table 5-3 *Comparison of the levels of the angiogenesis factors between the different age groups in the Control pre-operation datasets (p-values as determined by the Student's paired t-test).*

	0-39 *vs.* 40-69	0-39 *vs.* 70-100	40-69 *vs.* 70-100
Control VEGF (Pre-op)	0.775	0.463	0.175
Control PDGF-BB (Pre-op)	0.688	0.866	0.519
Control Follistatin (Pre-op)	0.097	0.341	0.155

Table 5-4 *Comparison of the VEGF levels between the different age groups.*

VEGF (Pre)	0-39	40-69	70-100	VEGF (Post)
0-39		0.3787	0.1598	0-39
40-69	0.4235		**<0.05**	40-69
70-100	0.3975	0.9078		70-100

p-values as determined by the Student's paired t-test. Significant values are shown in bold. White cells represent preoperative p values; light grey cells represent postoperative values.

Table 5-5 *Comparison of the PDGF-BB levels between the different age groups.*

PDGF-BB (Pre)	0-39	40-69	70-100	PDGF-BB (Post)
0-39		0.6605	0.8527	0-39
40-69	0.8237		0.2722	40-69
70-100	0.4933	0.2265		70-100

p-values as determined by the Student's paired t-test. White cells represent preoperative p values; light grey cells represent postoperative values.

Table 5-6 *Comparison of the Follistatin levels between the different age groups.*

Follistatin (Pre)	0-39	40-69	70-100	Follistatin (Post)
0-39		0.4412	0.9576	0-39
40-69	0.1006		**<0.05**	40-69
70-100	0.978	0.1709		70-100

p-values as determined by the Student's paired t-test. Significant values are shown in bold. White cells represent preoperative p values; light grey cells represent postoperative values.

Effect of gender on angiogenesis factors levels pre- and post-operatively

Finally, it was investigated whether gender had an effect on the levels of angiogenesis factors pre- and post-operatively as well as between the tumour patients and the Controls. This was achieved by grouping the patients based on their gender. The levels of the angiogenesis factors between the different gender groups were compared using the Student's paired t-test. The results of the analysis are shown in Table 5-7 and statistically significant results are shown in Figure 5-29, Figure 5-30, Figure 5-31, Figure 5-32, Figure 5-33 and Figure 5-34.

Table 5-7 ***Comparison of pre- and post-operative levels of angiogenesis factors VEGF, PDGF-BB and Follistatin and gender (p-values as determined by the Student's paired t-test). Significant values are shown in bold.***

		Angiogenesis factors		
		VEGF	**PDGF-BB**	**Follistatin**
All data *vs.* Male	Pre-op	0.955	0.720	0.895
	Post-op	0.306	0.734	0.403
All data *vs.* Female	Pre-op	0.947	0.672	0.828
	Post-op	0.301	0.702	0.416
Female *vs.* Male	Pre-op	0.917	0.504	0.762
	Post-op	0.086	0.535	0.170
Pre-op *vs.* Post-op	All Male	**<0.001**	**<0.001**	**<0.001**
Pre-op *vs.* Post-op	All Female	**0.001**	**<0.001**	**<0.001**
Male Control *vs.* Male Patient	Pre-op	0.825	**<0.001**	**<0.001**
Female Control *vs.* Female Patient	Pre-op	0.361	**<0.001**	**<0.001**
Male Control *vs.* Female Control	Pre-op	0.398	0.719	0.411

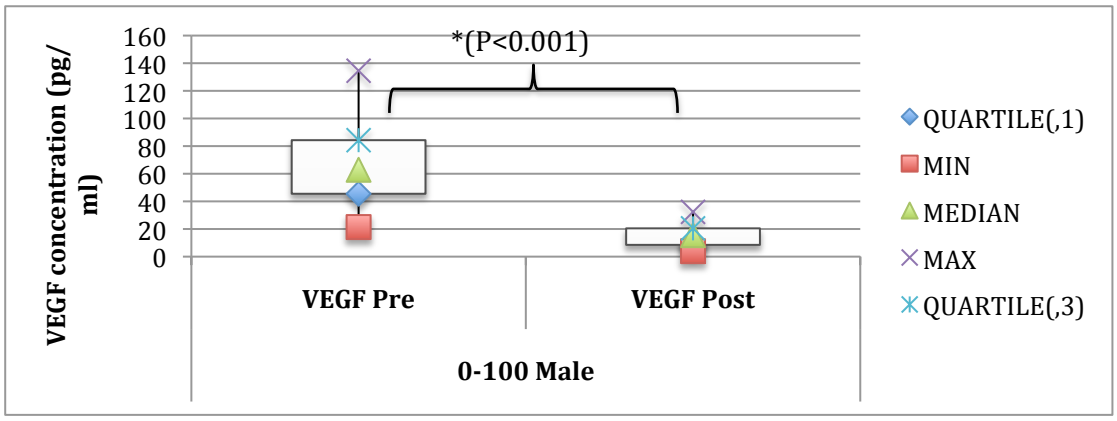

Figure 5-29 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

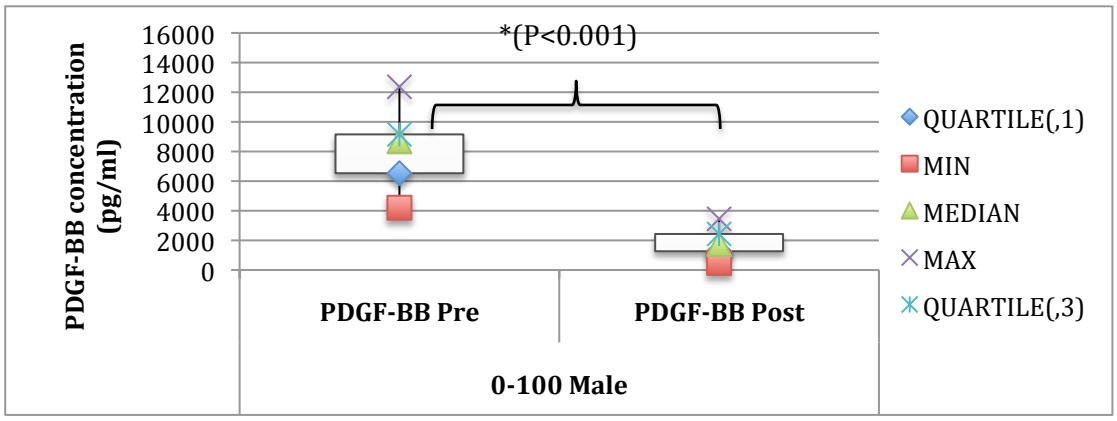

Figure 5-30 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

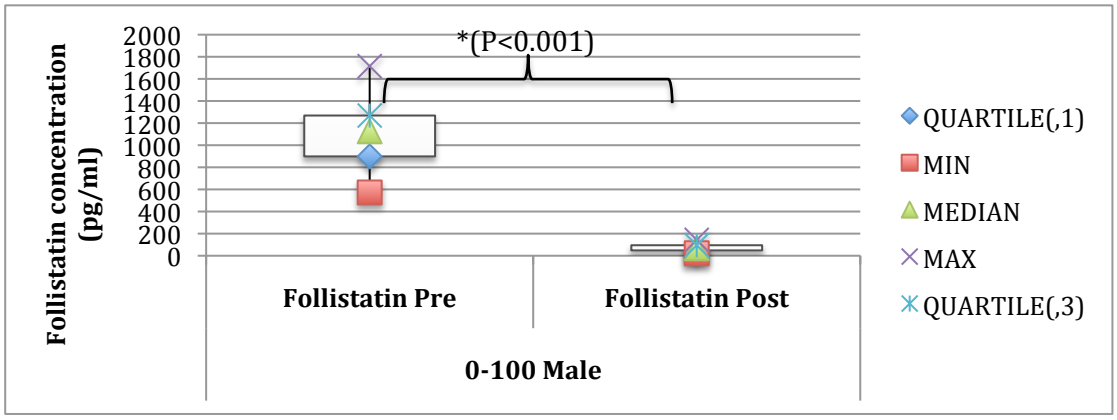

Figure 5-31 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the male malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

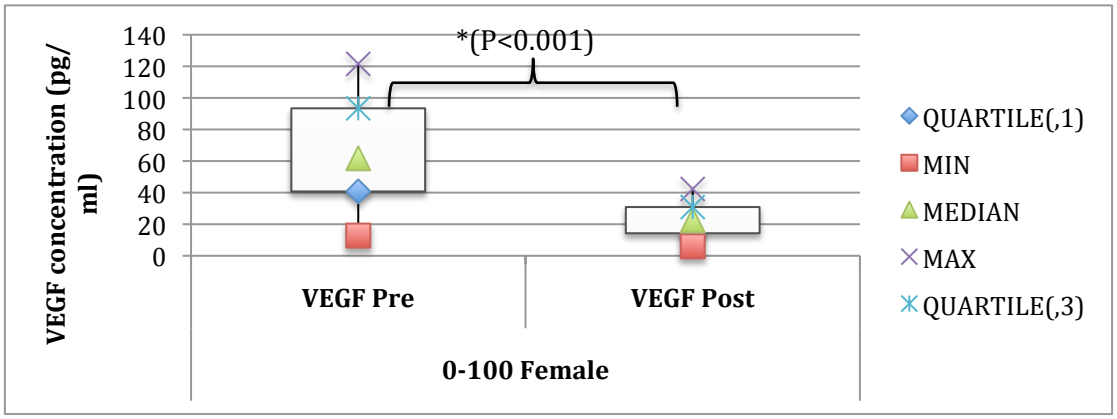

Figure 5-32 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of VEGF measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

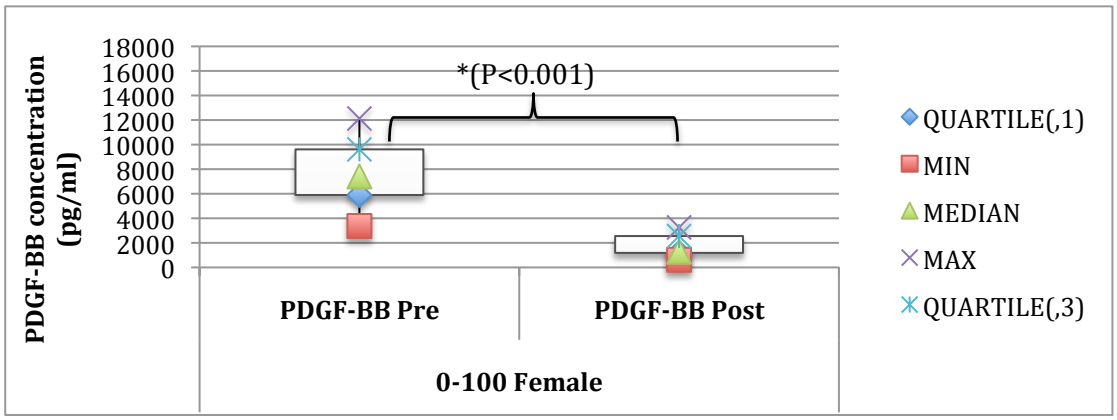

Figure 5-33 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of PDGF-BB measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

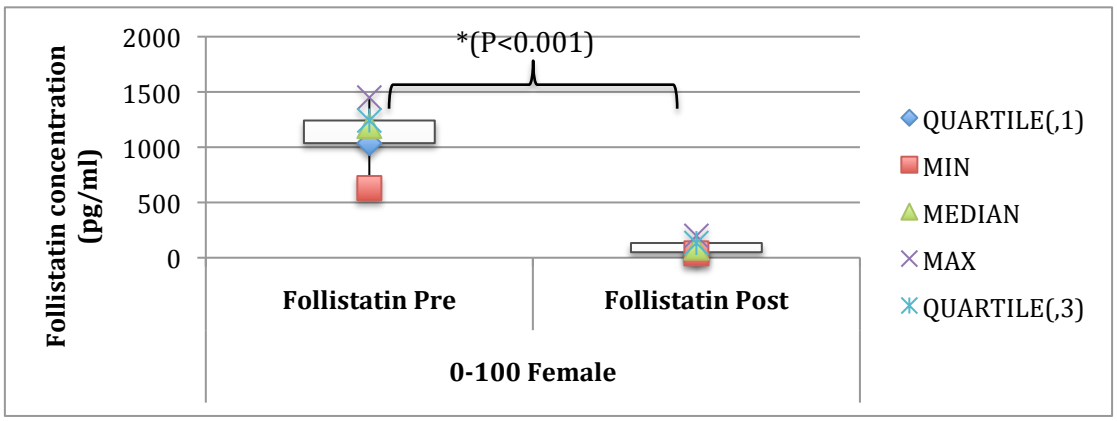

Figure 5-34 *Boxplot summarizing the pre and post-operative serum concentration (pg/ml) of Follistatin measured in the female malignant patients across all age groups. Asterisk (*) indicates statistical difference between datasets. P value shown in parenthesis.*

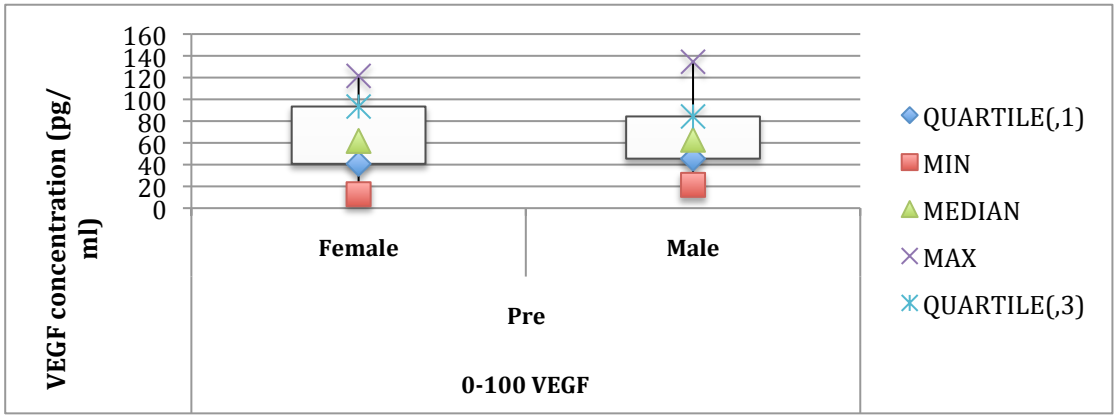

Figure 5-35 *Boxplot summarizing the preoperative serum concentration (pg/ml) of VEGF measured in the male and female malignant patients across all age groups*

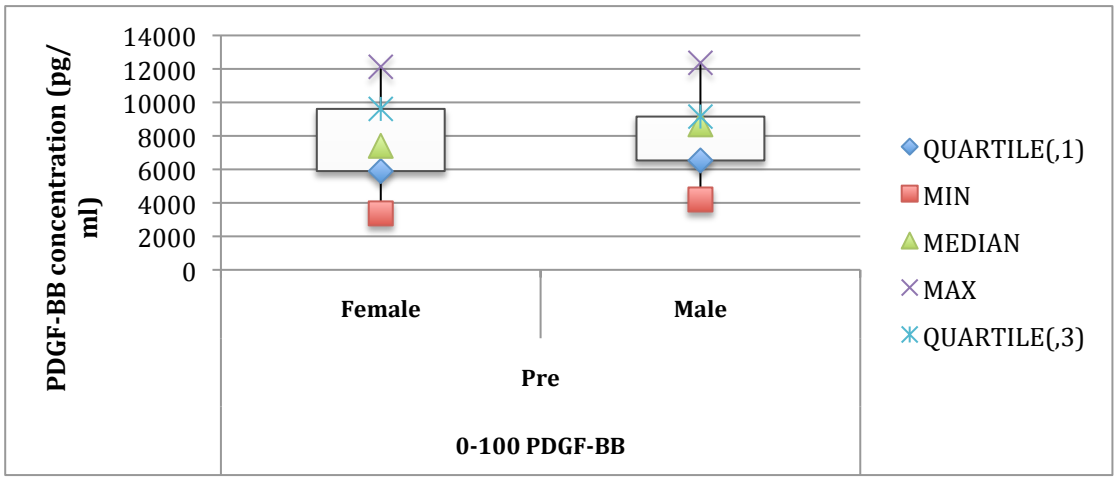

Figure 5-36 *Boxplot summarizing the preoperative serum concentration (pg/ml) of PDGF-BB measured in the male and female malignant patients across all age groups.*

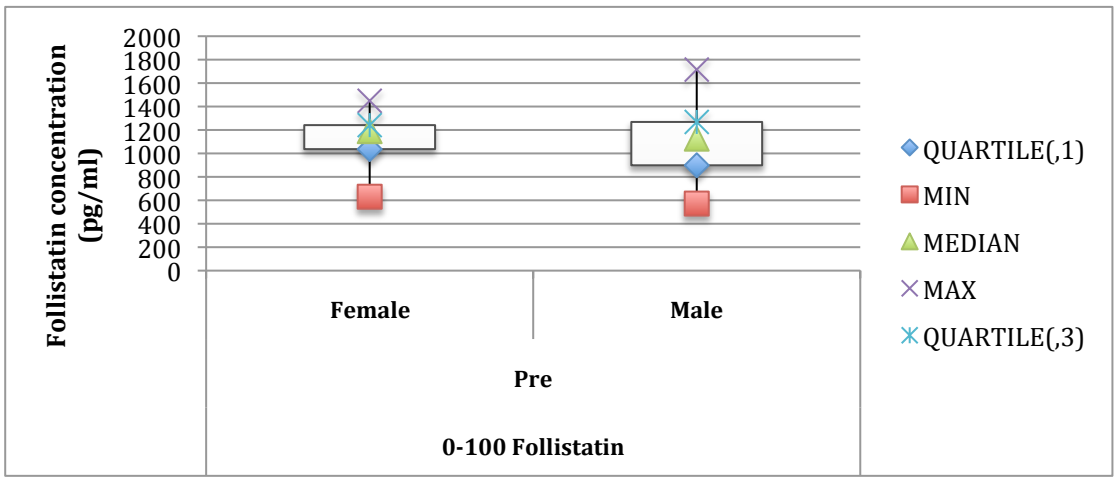

Figure 5-37 *Boxplot summarizing the pre-operative serum concentration (pg/ml) of Follistatin measured in the male and female malignant patients across all age groups.*

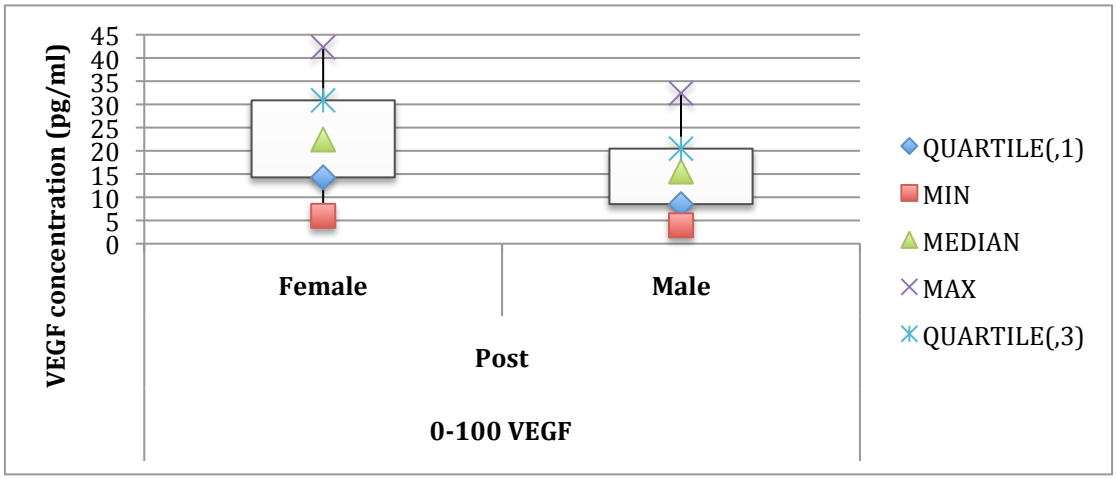

Figure 5-38 *Boxplot summarizing the post-operative serum concentration (pg/ml) of VEGF measured in the male and female malignant patients across all age groups.*

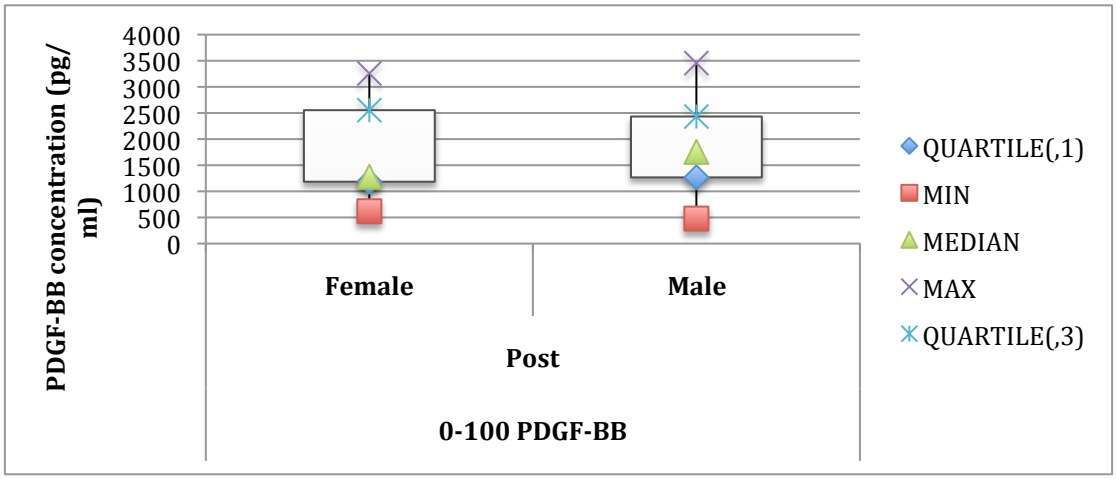

Figure 5-39 *Boxplot summarizing the post-operative serum concentration (pg/ml) of PDGF-BB measured in the male and female malignant patients across all age groups.*

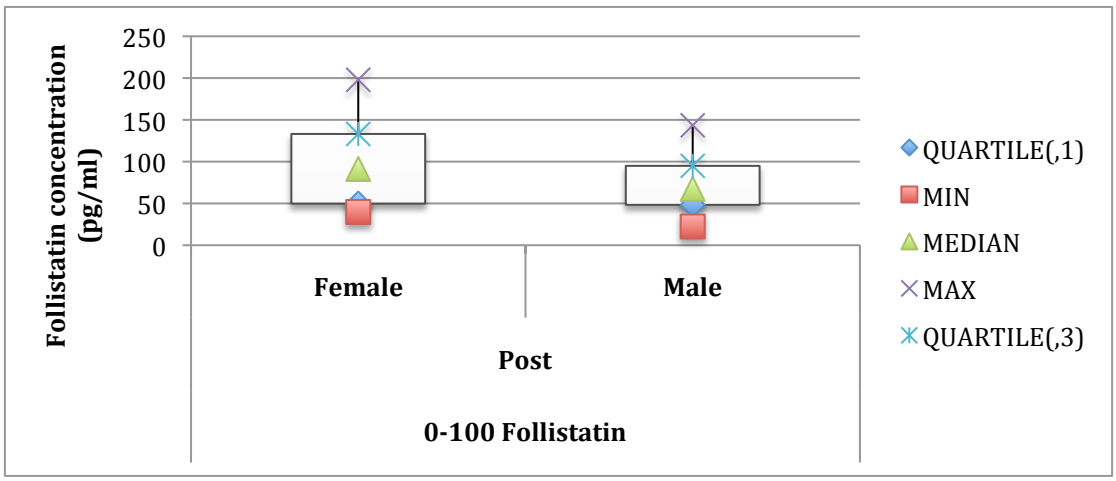

Figure 5-40 *Boxplot summarizing the post-operative serum concentration (pg/ml) of Follistatin measured in the male and female malignant patients across all age groups.*

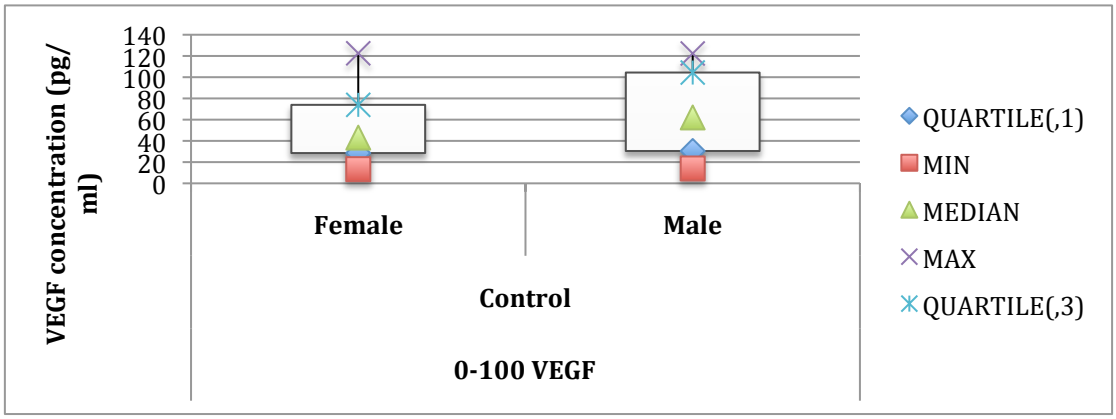

Figure 5-41 *Boxplot summarizing the serum concentration (pg/ml) of VEGF measured in the male and female controls across all age groups.*

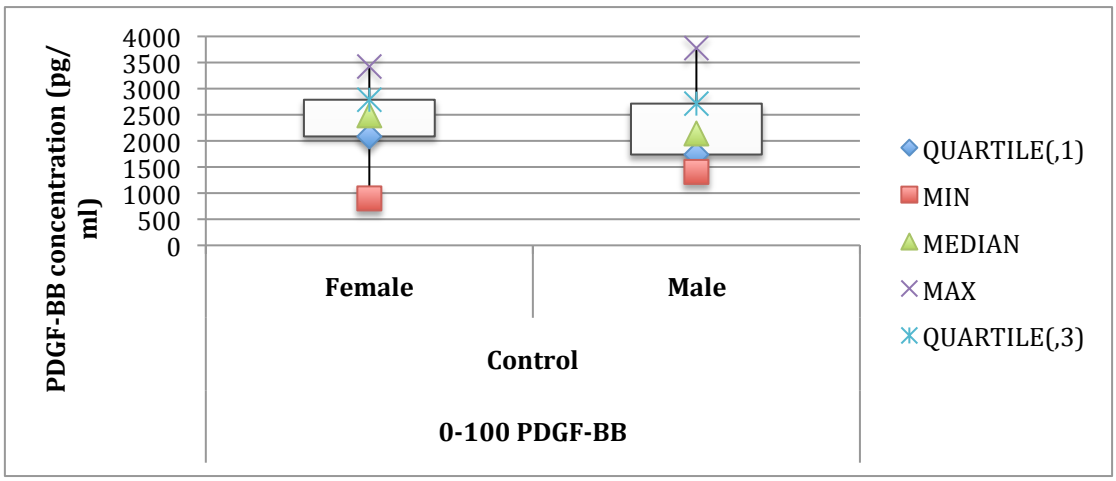

Figure 5-42 *Boxplot summarizing the serum concentration (pg/ml) of PDGF-BB measured in the male and female controls across all age groups.*

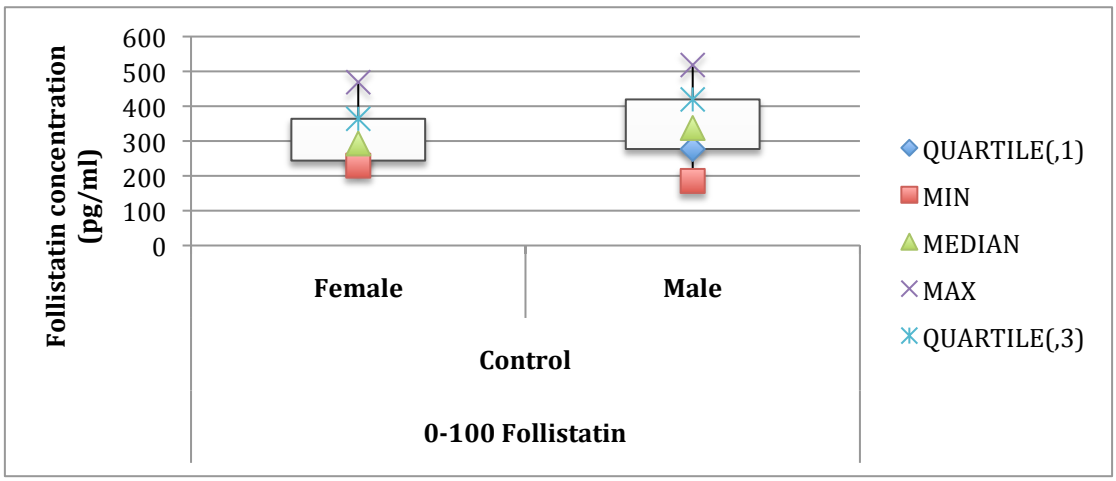

Figure 5-43 *Boxplot summarizing the serum concentration (pg/ml) of Follistatin measured in the male and female controls across all age groups.*

According to these results, neither the male nor the female group had displayed any significant difference against the combined dataset (males and females) as well as against the opposite gender (α=0.05) suggesting that gender doesn't seem to play a significant role for patients with glioblastoma tumour. These observations held true for both gender groups regardless of their operative stage (pre- *vs.* post-) (Figure 5-35, Figure 5-36, Figure 5-37, Figure 5-38, Figure 5-39 and Figure 5-40). On the contrary, when the operative stages were compared against each other for each gender group, both male and female groups had shown highly significant difference ($P<0.001$).

Similarly to the results above, gender didn't seem to have an effect in the preoperative Controls either (α=0.05) (*see Male Control vs. Female Control in Table 5.7* as well as Figure 5-41, Figure 5-42 and Figure 5-43). However, male and female Controls had significantly different levels only for the PDGF-BB and Follistatin when they were compared to their equivalent patient samples (Males $P<0.001$, Females $P<0.001$).

5.5 Discussion

The serum concentrations of three angiogenesis factors were measured from patients with malignant brain tumours as well as from controls affected by degenerative spinal disease. Out of these three factors, PDGF-BB and Follistatin were found to be lower in the controls compared to the preop patients. In addition, the preoperative levels of all three angiogenesis factors were higher than those observed postoperatively. Age and gender did not play a role on the angiogenesis factor concentrations.

There is a clear need for biomarkers to aid the management of malignant gliomas. VEGF and PDGF-BB have been previously proposed as potential candidates [214-219]. In agreement with the literature, both these molecules along with Follistatin have shown significant correlation with malignant gliomas in this present study. More specifically, the average concentrations found in the healthy population were for VEGF 61 (±37) pg/ml, for PDGF 2316 (±696) pg/ml and for Follistatin 335 (±93) pg/ml. These concentrations for VEGF and PDGF-BB are within the same range with those reported in the literature [233, 258]. This comparison bears the assumption that these concentrations are directly comparable and the assay used to measure them (i.e. Bio-Plex vs. ELISA) did not affect their values. There is evidence that angiogenesis factors (VEGF and PDGF) have a role in inducing the spontaneous resorption of the herniated disc disease [273, 274]. Given the fact that the healthy participants in this study were affected from benign spinal disease, the measured concentrations were expected to be higher than the reported normal average values. As it is unclear what was the past medical history of the participants used as controls in the aforementioned studies, it is difficult to draw any comparative conclusions.

Interestingly, Follistatin concentrations of the controls in this study were almost three times lower than the lowest concentrations reported in the literature [267]. This comes as a surprise as the controls were affected by degenerative

spinal disease and there is evidence to support that serum Follistatin concentrations reflect the severity of joint damage in osteoarthritis [275]. A possible explanation for this discrepancy might be that these concentrations are not directly comparable as the methodology used to measure them could be different. However, the BioRad assay used in the present study has clearly identified differences in the Follistatin concentrations measured between the two populations. This suggests that Follistatin does indeed differ between healthy controls and tumour patients and that this methodology is a reliable way for measuring that. The Bio-Plex assay was proven to be a suitable technique as it measures levels of multiple angiogenesis factors simultaneously from several samples within three to four hours. Furthermore, it is using a small quantity of sample, has low variability and high reproducibility making it appropriate for use in clinical practice.

Patients affected by malignant gliomas in this study had higher pre-operative concentrations for all three angiogenesis factors than the healthy participants. This is due to the fact that the tumour produces angiogenesis factors in response to hypoxia and in order to promote its neoangiogenesis and growth. Similar observations have also been reported in the literature [276-281]. Interestingly, the patients in this present study were taking Dexamethasone in high dose (16 mg/day), which is widely known to down regulate the production of VEGF, IL-6, Follistatin and PDGF-BB [276-281]. Despite this, significant difference between the two groups was observed. One could speculate that if the patients were not taking Dexamethasone, the measured concentrations would be even higher. Another possibility is that Dexamethasone did not down-regulate the production of angiogenesis factors in the tumor cells of these patients.

To summarise, patients affected by brain tumours have significantly higher concentrations of the three angiogenesis factors than the controls. Such finding supports the hypothesis that these molecules are suitable candidates to be used as biomarkers for the diagnosis of malignant gliomas.

The next thing investigated was the comparison of the angiogenesis factor concentrations between the patients affected by malignant glioma pre- and postoperatively. No studies have been found in the literature investigating the above. Angiogenesis factors that are related to the presence of the tumour would be expected to have their levels reduced following its removal. In the present study, a significant reduction of the concentrations of all three factors was observed postoperatively. A decrease of 78% (from 18 to 4 pg/ml) for VEGF, 77% (from 1950 to 450 pg/ml) for PDGF and 90% (from 295 to 30 pg/ml) for Follistatin was noted. The effect of debulking surgery is one of the possible explanatory factors for this observation. As tumour cells constitutively produce angiogenesis factors, cell removal by the operation would lead to a reduced production of these factors. Another cause for such drop could be the reduced hypoxic drive of the tumour following the operation. Hypoxia is known to stimulate angiogenesis factor production by tumour cells [214]. As surgery reduces the hypoxic area of the tumour, the stimulus for such production is also reduced.

Age did not influence the angiogenesis factor concentrations in any of the comparisons performed as all age groups tested yielded the same result. The sample size of the 0-39 years age group was small (less than 4) and this might explain the lack of significance between some of the compared datasets (see Table 5-2). Larger cohort of patients in this age group in future studies would strengthen the statistical significance of these findings. Moreover no significance was found between the age group comparisons within each dataset. No published reports on the effect of age on the angiogenesis factor concentrations of patients with malignant gliomas have been found. However, a study on healthy controls has reported similar findings to this current study [249].

According to the results of this study, gender similarly to age did not influence the concentrations of the angiogenesis factors tested in any of the datasets. As there are no previous reports on the patients with malignant gliomas this

finding is novel. However, studies on healthy individuals revealed that gender influenced VEGF levels [249] whereas Follistatin levels were unaffected [270].

This work demonstrates that all three tested angiogenesis factors are suitable candidates to use as biomarkers for malignant gliomas. Such biomarkers would allow clinicians to follow up patients during the course of their disease in terms of monitoring response to treatment as well as early detection of disease recurrence. While VEGF and PDGF-BB have already been proposed for this role, Follistatin is a novel biomarker for this disease. Another novelty of this work is that there are no previous studies investigating the pre vs. postoperative levels of angiogenesis factors in patients with malignant gliomas. As such, this work offers a significant contribution towards a better understanding of these molecules and their role in this disease. Further studies with larger cohort of healthy controls and patients would increase the reliability of the use of these molecules as biomarkers for malignant gliomas while it would allow novel ones to be discovered.

6 GENERAL DISCUSSION

Chemosensitivity testing was performed on twelve primary malignant glioma cultures at passage 0. The drugs tested are commonly administered chemotherapeutic drugs in clinical practice in our Unit. The results revealed that cultures derived from females or from patients under 65 years of age were more responsive to chemosensitivity testing. More importantly, the *in vitro* results showed correlation with the overall survival following chemotherapy of the donor patients. During chemosensitivity testing Temozolomide was found to have poor *in vitro* effect. Increased frequency of application as well as cell cycle synchronisation of glioma cell lines were found to improve the kill efficiency of Temozolomide.

Six molecules were considered as potential biomarkers. All of them were found to be effective at predicting the presence of malignant gliomas. With the exception of VEGF and PDGF-BB, this is the first report of these molecules as biomarkers for malignant gliomas. Overall age and gender of patients did not affect the biomarker results.

Glioblastoma is the most malignant tumor of central nervous system neoplasms with poor overall survival. Early detection and treatment of glioblastoma is challenging and little progress has been made so far. This imposes a clear need for improving the diagnosis and management of malignant gliomas. This study aimed to address both challenges and proposed ways of improvement.

The novelty of this study lies in the use of primary glioma cultures at passage 0 as opposed to short-term glioma cultures (passages 1-19) or glioma cell lines (20 passages or more). This type of culture at passage 0 is more suitable for chemosensitivity studies as it is more representative of the considerable genetic heterogeneity of malignant gliomas between patients. On the contrary, glioma

cell lines carry a more homogenous genetic load, as with every passage there is clonal selection of rapidly dividing cells. Such genetic misrepresentation in cell lines poses a limit as to their usefulness in research and clinical studies.

Another advantage of the present work is the availability of clinical data that enabled a retrospective evaluation of the chemosensitivity results. There was good correlation between *in vivo* and *in vitro* results for nine out of the twelve patients tested. *In vitro* chemosensitivity testing allowed the identification of the most suitable drug for each patient. This challenges the current clinical practice of administering Temozolomide to all patients regardless of their sensitivity to this drug. Introducing chemosensitivity testing for all patients undergoing chemotherapy treatment could potentially influence their clinical outcome.
Yet this hypothesis has to be validated by more chemosensitivity studies with larger patient numbers.

Molecular biomarkers are important in clinical practice as they can have diagnostic, predictive and prognostic potential in Glioblastomas. This work demonstrates that all six tested molecules are suitable candidates to use as biomarkers for malignant gliomas. Such biomarkers would allow clinicians to diagnose as well as follow up patients during the course of their disease in terms of monitoring response to treatment and early detection of disease recurrence.

Further studies would allow the discovery of even more biomarkers using similar high throughput screening assays. Association between those and chemosensitivity status could further enhance the management of malignant gliomas.

Reflecting on the learnings of this study a number of improvements could be proposed. Firstly, a greater number of specimens at different stages of the patients' disease would greatly reinforce the findings. Better coordination of the resources as well as more specialised personnel could facilitate this process.

Secondly, optimisation of the experimental design including frequency of drug administration, length of drug exposure and incubation, number of replicates and prevention of culture contamination would improve the efficiency and the sensitivity of the assay.

Patients' recruitment is a difficult and lengthy process. Moreover, availability of clinical data for research is not straightforward and their collection is not research oriented. Because of these two reasons prospective studies may run for many years. An alternative approach would be to use material already available in tumour banks and their readily accessible clinical data. Retrospective molecular analysis of these specimens using high throughput methods would allow the identification of the underlying genetic causes of the disease. Their subsequent correlation with clinical data could accelerate the translation of these findings to clinical practice.

Brain tumours represent 2% of all cancers in the UK. There is a growing interest in the scientific community and the charities towards research in brain tumours. The present work contributes to this effort. Scientific resources such as brain tumour tissue banks and clinical databases are invaluable assets in such research. Moreover, relevant online resources such as Brain Tumour Research continue raising awareness and attracting more funding. Coordination of these activities will lead to significant developments in this area in the years to come.

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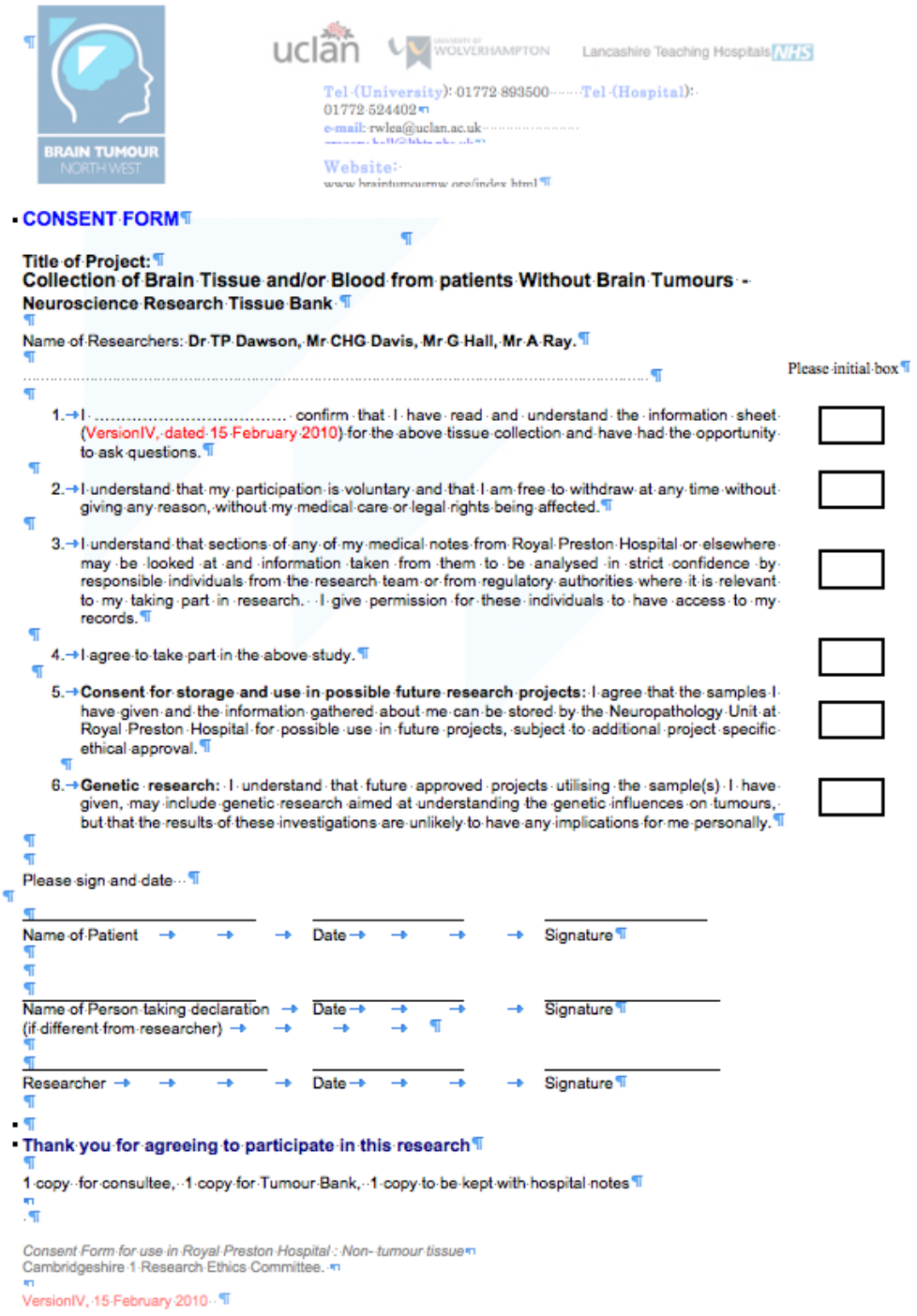
Appendices

Appendix 1

Consent forms and information sheets for patients and relatives



BRAIN TUMOUR NORTH WEST

uclan · WOLVERHAMPTON · Lancashire Teaching Hospitals NHS

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Website:

CONSENT FORM

Title of Project:
Collection of Brain Tissue and/or Blood from patients Without Brain Tumours - Neuroscience Research Tissue Bank

Name of Researchers: **Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.**

Please initial box

1. I ..... confirm that I have read and understand the information sheet (VersionIV, dated 15 February 2010) for the above tissue collection and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes from Royal Preston Hospital or elsewhere may be looked at and information taken from them to be analysed in strict confidence by responsible individuals from the research team or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

5. **Consent for storage and use in possible future research projects:** I agree that the samples I have given and the information gathered about me can be stored by the Neuropathology Unit at Royal Preston Hospital for possible use in future projects, subject to additional project specific ethical approval. ☐

6. **Genetic research:** I understand that future approved projects utilising the sample(s) I have given, may include genetic research aimed at understanding the genetic influences on tumours, but that the results of these investigations are unlikely to have any implications for me personally. ☐

Please sign and date

Name of Patient	Date	Signature
Name of Person taking declaration (if different from researcher)	Date	Signature
Researcher	Date	Signature

Thank you for agreeing to participate in this research

1 copy for consultee, 1 copy for Tumour Bank, 1 copy to be kept with hospital notes

Consent Form for use in Royal Preston Hospital : Non- tumour tissue
Cambridgeshire 1 Research Ethics Committee.

VersionIV, 15 February 2010

BRAIN TUMOUR NORTH WEST

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Website:
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CONSENT FORM

Title of Project:
Collection of Nervous System Tumour Tissue and/or Blood for Future Research Use - Neuroscience Research Tissue Bank

Name of Researchers: **Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.**

Please initial box

1. I ..... confirm that I have read and understand the information sheet (VersionIV, dated 15 February 2010) for the above tissue collection and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

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Please sign and date...

Name of Patient	Date	Signature
Name of Person taking declaration (if different from researcher)	Date	Signature
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Title of Project:
Collection of Brain Tissue and/or Blood from patients Without Brain Tumours - Neuroscience Research Tissue Bank

Name of Researchers: **Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.**

Please initial box

1. I ..... have been consulted about ..... 's participation in this tissue collection. I confirm that I have read and understand the information sheet (VersionIV, dated 15 February 2010) for the above tissue collection and have had the opportunity to ask questions. ☐

2. In my opinion he / she would have no objection to donating to the above tissue collection. ☐

3. I understand that his / her participation is voluntary and that I can request his / her donation to be withdrawn from the tissue bank at any time without giving any reason, without his/ her medical care or legal rights being affected. ☐

3. I understand that sections of any of his / her medical notes from Royal Preston Hospital or elsewhere may be looked at and information taken from them to be analysed in strict confidence by responsible individuals from the research team or from regulatory authorities where it is relevant to taking part in research. I give permission for these individuals to have access to his / her records. ☐

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5. **Genetic research:** I understand that future approved projects utilising the sample(s) he / she has given, may include genetic research aimed at understanding the genetic influences on tumours, but that the results of these investigations are unlikely to have any implications for him / her personally. ☐

Please sign and date...

_____	_____	_____
Name of Consultee	Date	Signature

Relationship to Participant: _____

_____	_____	_____
Name of Person taking declaration (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Thank you for agreeing to participate in this research

1 copy for consultee, 1 copy for Tumour Bank, 1 copy to be kept with hospital notes

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Collection of Nervous System Tumour Tissue and/or Blood for Future Research Use - Neuroscience Research Tissue Bank

Name of Researchers: **Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.**

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3. I understand that sections of any of his / her medical notes from Royal Preston Hospital or elsewhere may be looked at and information taken from them to be analysed in strict confidence by responsible individuals from the research team or from regulatory authorities where it is relevant to taking part in research. I give permission for these individuals to have access to his / her records. ☐

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Please sign and date...

Name of Consultee	Date	Signature
Relationship to Participant: _____		
Name of Person taking declaration (if different from researcher)	Date	Signature
Researcher	Date	Signature

Thank you for agreeing to participate in this research

1 copy for consultee, 1 copy for Tumour Bank, 1 copy to be kept with hospital notes

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Patient Information Sheet

Collection of Nervous System Tissue and/or Blood for Future Research Use
Neuroscience Research Tissue Bank

Name of Researchers: Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 30 minutes. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

Thank you for reading this.

What is the purpose of this project?

We wish to collect and store ("bank") blood and tissue samples from patients with brain and spinal disorders to support future research into the cause, diagnosis, treatment and outcome of such disorders. Some of these studies may include genetic research aimed at understanding the way in which genes (molecules instructing cell division and growth) influence the behaviour of these disorders. Researchers in Preston and elsewhere will be able to access the tissue and blood collection subject to ethical approval.

Why have I been chosen?

You are being asked to take part in this project because you are being investigated for a disorder of the nervous system (brain, spinal cord, nerves, or pituitary gland). Your management includes surgery during which tissue is removed routinely for access, diagnosis or treatment. We would like to ask you whether you would be willing to allow this tissue and any blood samples to be included in the research collection (tissue bank).

Do I have to take part?

NO, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your management and treatment will not be influenced in any way whether you wish to take part or not.

What will happen to me if I take part?

- Participating in this study by donating samples to the tissue bank will not affect your treatment in any way. The length of your operation and stay in hospital will not be affected and no additional surgery will be performed.
- We will ask you to give us permission (signed consent) to include samples removed as part of your surgery in our studies. It will not involve taking any tissue additional to that routinely removed for access, diagnostic or treatment purposes.
- Tissue will only be stored for research use once all diagnostic needs have been met.

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- You will be asked to give a blood sample at various times during your treatment in order to check how your treatment is affecting you. If you give permission for a blood sample to be stored for research extra blood will be taken on some of these occasions, but no additional venepuncture will be required.
- We will ask you for permission to consult your medical records at *Royal Preston Hospital* (or other relevant medical records elsewhere) for some information relevant to your illness.
- This information will include your age, gender, type and site of surgery, pathology diagnosis, epilepsy history, radiological (X-ray) features, your medical treatment and the response to treatment.

If you agree to take part in this study, we will have all that we need for our research and will not need to contact you again. Blood and archival tissue samples will be treated as gifts to research and held under the care of Brain Tumour North West, within the usual diagnostic archive, in accordance with Human Tissue Authority regulation.

What are the possible disadvantages and risks of taking part?
There are no disadvantages or risks to taking part.

What are the possible benefits of taking part?
There are no specific benefits to you directly, but the results of investigations using this tissue may help others with similar disorders.

- **What happens to tissue in the bank?**

Tissue will be securely stored until allocated to an ethically approved project. During analysis some will be "used up". This allocation will go on until the supply is exhausted.

Will my taking part in this study be kept confidential?
YES. If you agree to take part in this study, only the relevant information mentioned above will be extracted from your records. Any information about you released by the Tissue Bank will have personal details such as your name and address removed from it. The same will apply to the tissue samples used in any laboratory studies. The information and tissues will only be known by a research number, which will prevent researchers from knowing your identity.

- **What will happen if I don't want to carry on with the study?**

Any unused tissue or blood stored in the Tissue Bank will be tranferred to the diagnostic archive and stored or disposed according to departmental diagnostic protocols. No further allocations will be made to research projects. Data from previously allocated tissue or blood may already exist and will remain associated with those projects anonymised to the researcher.

- **Will any genetic tests be done?**

DNA derived from tissue may be examined for abnormalities, which may give information on the cause of a disorder. It is unlikely to produce results with a direct influence on you or your relatives.

BRAIN TUMOUR NORTH WEST

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- **What happens if something goes wrong?**

The planned research will have no influence on your treatment. The banking of your tissue or blood for research carries no risk. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated, the normal National Health Service complaints mechanisms will be available to you.

What will happen to the results of the research study?

Results will be presented at conferences and published as scientific papers, but you will not be identified in any report or publication. Results obtained from your specimens are unlikely to include information of immediate clinical relevance, but should anything helpful be found, this will be conveyed to your treatment team.

- **Who is organising and funding the research?**
- The neuroscience tissue bank is organised by neuropathologists, neurosurgeons, oncologists and researchers at Royal Preston Hospital. Funding for tissue banking is obtained from Neuroscience Charitable Funds and from ongoing project funding. None of the members of the brain tumour tissue banking team are being paid for their participation in the project. Future research studies utilising banked tissues will be funded by a variety of funds/charities.

- **Who has reviewed the study?**

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by Cambridgeshire 1 Research Ethics Committee.

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you very much for reading this information sheet.

Contacts for Further Information

Mr Gregory Hall
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Consultee Information Sheet

Collection of Nervous System Tissue and/or Blood for Future Research Use
Neuroscience Research Tissue Bank

Name of Researchers: Dr TP Dawson, Mr CHG Davis, Mr G Hall, Mr A Ray.

We believe ..... is unable to decide for him/herself whether he/she wants to join the study, so we are asking you to advise whether you feel he/she would have wanted to take part. We ask you to consider the following information about the study and what you know of his/her wishes and feelings about research. We would like to know whether or not you feel he/she would have agreed to join the study if he/she had been able to decide. If you feel unable to give advice about this, please say so.

Thank you for reading this.

What is the purpose of this project?

We wish to collect and store ("bank") blood and tissue samples from patients with brain and spinal disorders to support future research into the cause, diagnosis, treatment and outcome of such disorders. Some of these studies may include genetic research aimed at understanding the way in which genes (molecules instructing cell division and growth) influence the behaviour of these disorders. Researchers in Preston and elsewhere will be able to access the tissue and blood collection subject to ethical approval.

Why has he/she been chosen?

He/she is being asked to take part in this project because he/she is being investigated for a disorder of the nervous system (brain, spinal cord, nerves, or pituitary gland). His/her management includes surgery during which tissue is removed routinely for access, diagnosis or treatment. We would like to ask you whether you would be willing to allow this tissue and any blood samples to be included in the research collection (tissue bank).

Does he/she have to take part?

- NO, it is up to you to decide on his/her behalf whether or not to take part. If you do decide he/she would have liked to take part you will be given this information sheet to keep and be asked to sign a declaration form. If you decide he/she would have liked to take part you are still free to withdraw that permission at any time and without giving a reason. His /her management and treatment will not be influenced in any way whether you decide he/she should take part or not.

What will happen to him/her if he/she takes part?

- Participating in this study by donating samples to the tissue bank will not affect his/her treatment in any way. The length of his/her operation and stay in hospital will not be affected and no additional surgery will be performed.

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Website:
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- We will ask you to give us permission (signed declaration) to include samples removed as part of his/her surgery in our studies. It will not involve taking any tissue additional to that routinely removed for access, diagnostic or treatment purposes.
- Tissue will only be stored for research use once all diagnostic needs have been met.
- He/she will be asked to give a blood sample at various times during his/her treatment in order to check how his/her treatment is affecting him/her. If you give permission for a blood sample to be stored for research extra blood will be taken on some of these occasions, but no additional venepuncture will be required.
- We will ask you for permission to consult his/her medical records at *Royal Preston Hospital* (or other relevant medical records elsewhere) for some information relevant to his/her illness.
- This information will include his/her age, gender, type and site of surgery, pathology diagnosis, epilepsy history, radiological (X-ray) features, his/her medical treatment and the response to treatment.

If you agree to him/her taking part in this study, we will have all that we need for our research and will not need to contact you again. Blood and archival tissue samples will be treated as gifts to research and held under the care of Brain Tumour North West, within the usual diagnostic archive, in accordance with Human Tissue Authority regulation.

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- **What will happen if I don't want him/her to carry on with the study?**

Any unused tissue or blood stored in the Tissue Bank will be tranferred to the diagnostic archive and stored or disposed according to departmental diagnostic protocols. No further allocations will be made to research projects. Data from previously allocated tissue or blood may already exist and will remain associated with those projects anonymised to the researcher.

- **What will happen if he/she recovers the capacity to consent to the study?**
  He/she will be shown the patient information sheet and one of the researchers will discuss the study with him/her. He/she will be given the opportunity to consent to participation in the study him/herself or to withdraw from the study.

- **Will any genetic tests be done?**
  DNA derived from tissue may be examined for abnormalities, which may give information on the cause of a disorder. It is unlikely to produce results with a direct influence on him/her or his/her relatives.

- **What happens if something goes wrong?**
  The planned research will have no influence on his/her treatment. The banking of his/her tissue or blood for research carries no risk. Regardless of this, if you wish to complain about any aspect of the way you, or he/she, have been approached or treated, the normal National Health Service complaints mechanisms will be available to you.

What will happen to the results of the research study?
Results will be presented at conferences and published as scientific papers, but he/she will not be identified in any report or publication. Results obtained from his/her specimens are unlikely to include information of immediate clinical relevance, but should anything helpful be found, this will be conveyed to his/her treatment team.

- **Who is organising and funding the research?**
- The neuroscience tissue bank is organised by neuropathologists, neurosurgeons, oncologists and researchers at Royal Preston Hospital. Funding for tissue banking is obtained from Neuroscience Charitable Funds and from ongoing project funding. None of the members of the brain tumour tissue banking team are being paid for their participation in the project. Future research studies utilising banked tissues will be funded by a variety of funds/charities.

- **Who has reviewed the study?**
  All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect his/her interests. This study has been reviewed and given a favourable opinion by Cambridgeshire 1 Research Ethics Committee.

You will be given a copy of the information sheet and a signed declaration form to keep.

Thank you very much for reading this information sheet.

Contacts for Further Information

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Dr Timothy Dawson
Consultant Neuropathologist
Royal Preston Hospital
e-mail: Timothy.Dawson@lthtr.nhs.uk
01772 522147

Appendix 2

Cell culture media constituents

Cell Culture Media Constituents (Sigma – Aldrich)		
	DMEM*	HAM's F-12
Contents	g/l	g/l
L-Arginine HCL	0.084	0.211
L-cysteine 2 HCL	0.0626	0.035
L-Glutamine	0.584	0.146
G;ycine	0.03	0.00751
L-Histidine HCL	0.042	0.02096
L-isoleucine	0.105	0.00394
L-leucine	0.105	0.0131
L-lysine HCL	0.146	0.0365
L-methionine	0.03	0.00448
L-phenylalanine	0.066	0.00496
L-Proline	-	0.0345
L-serine	0.042	0.0105
L-threonine	0.095	0.0119
L-tryptophan	0.016	0.00204
L-tyrosine	0.1038	0.00778
L-valine	0.094	0.0117
L-biotin	-	0.0000073
Choline chloride	0.004	0.01396
Folic acid	0.004	0.00132
Myo-inositol	0.0072	0.018
Niacinamide	0.004	0.000037
D=pantothenic acid	0.004	0.00048
Pyridoxal-HCL	0.004	0.000062
Riboflavin	0.0004	0.000038
Thiamine-HCL	0.004	0.00034
Calcium chloride	0.2	0.0333

Cupric sulphate	-	0.0000025
Ferrous sulphate	-	0.000834
Ferric nitrate	0.0001	-
Magnesium sulphate	0.098	-
Magnesium chloride	-	0.0576
Potassium chloride	0.4	0.224
Sodium chloride	6.4	7.599
Sodium phosphate	0.109	0.14204
Glucose	4.5	1.802
Phenol red-Na	0.0159	0.0013
Vitamin B12	-	0.00136
Hypoxanthine	-	0.00408
Linoleic acid	-	0.000084
Putrescien di HCL	-	0.000161
Pyruvic acid-Na	-	0.11
Thymidine	-	0.00073
*DMEM- Dulbeco's Modified Eagle's Medium		

Appendix 3

Number of patients in the cytokines and angiogenesis factors analysis

	Age	IL-6	IL-8	IL-10
Control	0-100	20	20	21
	0-39	3	4	3
	40-69	11	11	12
	70-100	6	5	6
Pt preop	0-100	26	32	27
	0-39	2	4	2
	40-69	13	14	14
	70-100	11	14	11
Pt Postop	0-100	29	31	28
	0-39	4	3	3
	40-69	11	15	13
	70-100	14	13	12

Table 1: Summary of number of patients included in the analysis for each of the datasets grouped by age.

	Control	Pt Preop	Pt Postop
Female			
IL-6	8	10	11
IL-8	7	13	12
IL-10	9	9	8
Male			
Il-6	12	16	18
IL-8	13	19	19
IL-10	12	18	20

Table 2: Summary of number of patients included in the analysis for each of the datasets grouped by gender.

	Age	VEGF	PDGF-BB	Follistatin
Controls	0-100	27	23	23
	0-39	4	2	2
	40-69	16	14	14
	70-100	7	7	7
Pt preop	0-100	31	29	29
	0-39	3	3	4
	40-69	15	13	13
	70-100	13	13	12
Pt postop	0-100	28	19	30
	0-39	3	3	3
	40-69	15	14	14
	70-100	9	11	13

Table 3: Summary of number of patients included in the analysis for each of the datasets grouped by age.

	Control	Pt Preop	Pt Postop
Female			
VEGF	10	13	12
PDGF-BB	9	12	13
Follistatin	10	13	12
Male			
VEGF	17	18	16
PDGF-BB	14	17	16
Follistatin	14	18	17

Table 4: Summary of number of patients included in the analysis for each of the datasets grouped by gender.

	Age	IL-6	IL-8	IL-10
Control	0-100	20	20	21
	0-39	3	4	3
	40-69	11	11	12
	70-100	6	5	6
Pt preop	0-100	26	32	27
	0-39	2	4	2
	40-69	13	14	14
	70-100	11	14	11
Pt Postop	0-100	29	31	28
	0-39	4	3	3
	40-69	11	15	13
	70-100	14	13	12

Table 5: Summary of number of patients included in the analysis for each of the datasets grouped by age.

	Control	Pt Preop	Pt Postop
Female			
IL-6	8	10	11
IL-8	7	13	12
IL-10	9	9	8
Male			
Il-6	12	16	18
IL-8	13	19	19
IL-10	12	18	20

Table 6: Summary of number of patients included in the analysis for each of the datasets grouped by gender.

	Age	VEGF	PDGF-BB	Follistatin
Controls	0-100	27	23	23

	0-39	4	2	2
	40-69	16	14	14
	70-100	7	7	7
Pt preop	0-100	31	29	29
	0-39	3	3	4
	40-69	15	13	13
	70-100	13	13	12
Pt postop	0-100	28	19	30
	0-39	3	3	3
	40-69	15	14	14
	70-100	9	11	13

Table 7: Summary of number of patients included in the analysis for each of the datasets grouped by age.

	Control	Pt Preop	Pt Postop
Female			
VEGF	10	13	12
PDGF-BB	9	12	13
Follistatin	10	13	12
Male			
VEGF	17	18	16
PDGF-BB	14	17	16
Follistatin	14	18	17

Table 8: Summary of number of patients included in the analysis for each of the datasets grouped by gender.

Appendix 4

Tables with raw data. Highlighted values indicate outliers.

Appendix Table 1

				pre	Post
Age Group	**Cytokine**	**Age**	**Sex**	**IL-6**	**IL-6**
0-39	446	30	M	15.32	10.73
	375	35	F	0	29.26
	438	39	F	0	14.87
	532	39	F	70.62	1.82
40-69	641	44	M	0	10.38
	665	46	M	0	9
	378	46	F	0	6.86
	443	46	M	1.24	44.32
	550	50	F	0	16.93
	423	51	M	22.22	18.28
	455	53	M	0	555.92
	545	58	M	0	51.11
	536	63	F	6.3	2.11
	555	63	F	0	1.44
	413	63	M	0	8.66
	516	66	M	0	177.44
	511	67	M	0	4.47
	405	67	M	0	29.13
	534	67	M	0	26.18
	440	69	F	0	195.05
70-100	548	70	M	0	29.65
	437	70	F	0	12.13
	402	70	M	0.7	7.08
	605	72	M	10.74	27.71
	501	73	F	0	4.26
	628	73	M	8.57	12.25
	459	73	F	13.66	8.77
	434	74	M	0	19.39
	523	75	M	0	8.21
	385	76	F	0	11.66
	403	76	M	1.94	10.61
	373	76	M	0	3.53
	471	76	M	0	20.5
	391	80	M	0	2.91
	415	80	F	0	55.93

Appendix Table 2

				pre	post
Age Group	**Cytokine**	**Age**	**Sex**	**IL-8**	**IL-8**
	446	30	M	4.92	3.47
	375	35	F	1.46	1042.11
	438	39	F	1.57	4.17
0-39	532	39	F	5.34	29.84
	641	44	M	3.43	15.57
	665	46	M	1.23	1.48
	378	46	F	1.68	19.99
	443	46	M	1.01	5.12
	550	50	F	1.68	18.32
	423	51	M	35.58	24.31
	455	53	M	3.43	75.14
	545	58	M	3.21	9.14
	536	63	F	6.61	12.35
	555	63	F	2.45	16.41
	413	63	M	2.12	5.23
	516	66	M	3.64	29.24
	511	67	M	4.92	17.84
	405	67	M	11.89	12.47
	534	67	M	2.78	11.52
40-69	440	69	F	0.78	38.52
	548	70	M	7.02	27.43
	437	70	F	4.28	9.5
	402	70	M	1.9	1746.77
	605	72	M	4.71	15.69
	501	73	F	2.89	3.35
	628	73	M	1.35	9.02
	459	73	F	1.46	6.65
	434	74	M	19.55	23.47
	523	75	M	4.5	16.88
	385	76	F	0.78	9.26
	403	76	M	2.34	1850.29
	373	76	M	7.23	7.24
	471	76	M	5.13	11.64
	391	80	M	2.34	6.3
70-100	415	80	F	4.5	15.45

Appendix Table 3

				pre	post
Age Group	**Cytokine**	**Age**	**Sex**	**IL-10**	**IL-10**
	446	30	M	7.3	6
	375	35	F	0	25.34
	438	39	F	1.53	6.37
0-39	532	39	F	110.02	5.91
	641	44	M	0	5.17
	665	46	M	0.43	2.91
	378	46	F	0.47	25.46
	443	46	M	0	5.08
	550	50	F	0	3.73
	423	51	M	620.79	10.26
	455	53	M	0.34	121.89
	545	58	M	2.11	12.94
	536	63	F	5.69	8.58
	555	63	F	0	2.53
	413	63	M	1.43	4.44
	516	66	M	0	3.38
	511	67	M	1.62	8.87
	405	67	M	0	3.34
	534	67	M	0	10.41
40-69	440	69	F	0	19.66
	548	70	M	0.3	14.03
	437	70	F	5.16	6
	402	70	M	0.09	9.51
	605	72	M	154.54	7.85
	501	73	F	1.1	8.29
	628	73	M	11.3	4.89
	459	73	F	65.53	2.33
	434	74	M	2.7	6.51
	523	75	M	0	4.99
	385	76	F	1.57	23.06
	403	76	M	0	16.92
	373	76	M	0	7.9
	471	76	M	0	4.18
	391	80	M	0.02	5.72
70-100	415	80	F	2.9	19.07

Appendix Table 4

				pre	post
Age Group	**Angio**	**Age**	**Sex**	**VEGF**	**VEGF**
0-39	374	35	F	61.76	42.32
	427	35	F	12.8	30.2
	436	39	F	218.24	77.52
	428	39	F	73.96	15.08
40-69	640	44	M	138.88	32.4
	660	46	M	47.16	20.56
	472	46	F	55.8	23.56
	442	46	M	22.96	9.12
	418	50	F	121.56	32.88
	421	51	M	88.24	23.56
	453	53	M	64.52	15.44
	545	58	M	76.08	18.16
	535	63	F	106.2	21.32
	552	63	F	86.36	38.64
	411	63	M	33.8	7.44
	515	66	M	127.2	30.44
	510	67	M	44.96	14.36
	404	67	M	52.24	3.96
	533	67	M	73.96	20.24
	439	69	F	27.6	13.32
70-100	544	70	M	111.72	54
	435	70	F	111.96	71.72
	396	70	M	134.56	87.6
	597	72	M	37.16	6.4
	500	73	F	40.76	6
	626	73	M	61.2	8.88
	458	73	F	47.72	11.88
	522	75	M	57.24	15.64
	382	76	F	93.4	24
	397	76	M	84.44	102.92
	371	76	M	21.4	4.64
	390	80	M	83.84	20.44
	410	80	F	25.96	14.6

Appendix Table 5

				pre	post
Age Group	**Angio**	**Age**	**Sex**	**PDGF-BB**	**PDGF-BB**
0-39	374	35	F	9501.12	2626.32
	427	35	F	151.16	5972.6
	436	39	F	5371.12	1270.16
	428	39	F	12126.6	1022.4
40-69	640	44	M	8902.96	2451.92
	660	46	M	5604.36	1906.12
	472	46	F	5968.44	1280.32
	442	46	M	792.32	938.24
	418	50	F	9943.4	1184.44
	421	51	M	12122.2	2918.08
	453	53	M	7178.92	1514
	545	58	M	9067.88	1711.16
	535	63	F	11056.48	2553.64
	552	63	F	6887.76	3257.12
	411	63	M	9153.32	2426.04
	515	66	M	12364.32	2633.48
	510	67	M	5536.68	1798.12
	404	67	M	6533.12	478.04
	533	67	M	19516.56	6269.56
	439	69	F	806.16	1506.56
70-100	544	70	M	9025.88	3455.48
	435	70	F	7925.64	2598.76
	396	70	M	10711.64	2216.72
	597	72	M	6913.32	825.72
	500	73	F	5741.4	661.04
	626	73	M	7216.28	1461.76
	458	73	F	7958.52	1332.72
	522	75	M	4202.08	1064.12
	382	76	F	5951.04	1217.12
	397	76	M	8689.08	4306.4
	371	76	M	11260.8	4228.92
	390	80	M	6480.04	1334.72
	410	80	F	3371.08	618.8

Appendix Table 6

				pre	post
Age Group	**Angio**	**Age**	**Sex**	**Follistatin**	**Follistatin**
0-39	374	35	F	1318.44	228.76
	427	35	F	1201.72	102.68
	436	39	F	1221.84	168.88
	428	39	F	1094.72	39.76
40-69	640	44	M	857.96	57.64
	660	46	M	854.56	60.6
	472	46	F	630.52	91.24
	442	46	M	799.44	35.28
	418	50	F	1119.28	49.88
	421	51	M	915.84	67.32
	453	53	M	1715.24	73.92
	545	58	M	1270.6	48.32
	535	63	F	2245.88	125.64
	552	63	F	784.88	82.12
	411	63	M	1120.44	52.4
	515	66	M	1120.44	259.48
	510	67	M	1911.88	143.52
	404	67	M	893.08	22.36
	533	67	M	1459.88	213.56
	439	69	F	2370.68	43.08
70-100	544	70	M	1255.12	127.28
	435	70	F	1261.08	133.12
	396	70	M	1670.36	69.88
	597	72	M	1131	140.28
	500	73	F	1189.88	77.08
	626	73	M	1182.8	74.68
	458	73	F	978.8	45.76
	522	75	M	1503.84	47.64
	382	76	F	1448.92	137.4
	397	76	M	572.08	94.96
	371	76	M	1262.28	126.36
	390	80	M	1022.64	46.56
	410	80	F	2113.24	197.96

Appendix Table 7

BTNW	Age	Gender	Control-IL-6	Control-IL-8	Control-IL-10
574	26	F	2.31	2.74	1.71
575	68	F	1.83	3.17	0
579	100	F	0.26	3.17	2.34
580	81	F	0.42	4.12	0.01
582	75	F	0.18	5.42	0
583	39	M	8.92	3.88	14.66
585	35	M	18.85	2.74	17
586	61	M	0.34	8.59	0
587	65	F	0.11	4.64	0
589	45	M	3.31	13.05	3.42
591	85	M	0.42	3.68	0.11
592	68	F	0.73	9.92	1.34
593	84	M	1.51	5.07	0.91
594	57	M	1564.01	4.95	1617.42
595	39	M	2.31	3.7	6.26
600	68	M	1.67	6.03	4
601	61	M	0.26	1.72	0.34
603	87	M	0.26	24.55	0.08
606	37	M	3.43	111.39	4.32
607	56	F	7.26	19.85	6.21
608	48	F	3.56	32.16	3.37
609	60	M	2.56	12.67	3.14
610	58	M	3.5	15.94	2.21
616	65	M	1.35	3	0.18

Appendix Table 8

BTNW	Age	Gender	Control-VEGF	Control-PDGF-BB	Control-Folistatin
574	26	F	39.19	1904.01	258.05
575	68	F	56.26	898.63	229.42
576	81	M	62.08	2771.63	372.83
579	100	F	22	3086.74	330.21
580	81	F	33.64	2787.47	293.01
582	75	F	46.99	2084.97	243.84
583	39	M	122.33	3112.12	384.49
585	35	M	40.87	4495.45	799.46
586	61	M	72.82	3776.26	246.61
587	65	F	122.52	2655.5	417.97
589	45	M	22.02	1901.94	518.29
591	85	M	14	1587	308.62
592	68	F	13.36	2135.63	229.56
593	84	M	36.64	1405.69	308.62
594	57	M	117.62	2538.24	721.99
595	39	M	30.48	2231.73	185.36
598	68	F	79.85	2488.33	363.86
599	62	M	59.17	2415.75	431.11
600	68	M	116.22	2938.14	317.06
601	61	M	22.54	1727.2	470.77
603	87	M	104.39	1576.6	357.91
606	37	M	74.7	1773.37	242.79
607	56	F	88.47	3423.29	884.87
608	48	F	26.82	4592.99	468.93
609	60	M	74.88	6965.25	812.88
610	58	M	25.33	2051.71	266.63
616	65	M	112.88	4579.94	457.51

Appendix Table 9

BTNW No	Gender	Age	Cis100	Cis10	Cis1	Detergent	Medium
BTNW338	M	72	87453	124216	149778	965	155303
			73264	119370	108605	805	121672
			79451	124115	88722	615	96024
			70217	104853	122550	545	138431
BTNW370	F	66	41626	108291	158080	990	149317
			47978	118016	153151	940	161532
			46352	127616	147273	710	160378
			47376	119873	152918	690	163942
BTNW377	F	45	27314	33118	56360	605	77854
			23907	30902	51459	415	56712
			30150	39827	52122	485	50877
			27214	53196	50275	405	54040
BTNW382	F	75	63	13	353	193	43337
			113	123	35192	143	46950
			253	213	263	123	47472
			193	243	313	73	52452
BTNW500	F	73	75658	140386	140426	713	206563
			97597	118171	193408	1063	171810
			133957	147304	144391	1303	212774
			114629	118999	198404	1413	200009
BTNW533	M	67	170492	168749	255330	1233	301016
			184861	169935	325798	1423	281332
			190717	142904	281475	933	265587
			211432	113478	260912	873	303710
BTNW544	M	68	13487	174842	110505	350	47710
			22109	159419	106904	400	49156
			19905	107751	137258	460	52590
			16320	83709	112089	240	57773
BTNW546	F	50	34670	60196	108690	1173	156783
			55143	98254	127232	993	178935
			38140	77490	129182	1283	187823
			59242	110435	158595	1313	163396
BTNW590	M	50	34338	104188	88348	1048	107298
			27258	107298	129878	778	126408
			37898	103498	122228	1018	117618
			16088	98328	124328	1448	120438
BTNW597	M	71	20073	24583	59923	753	65103
			12443	25023	71513	573	66923
			9583	30293	66793	683	77783
			22083	35383	81663	343	70023
BTNW365	F	42	47634	217799	1687281	3265	344995
			55787	803650	1043007	4265	427367
			53196	790107	759881	4775	434793
			61895	768147	1182408	4035	450891
BTNW374	F	34	80459	774029	740398	4665	471293

			94737	904611	1114796	4425	443414
			80922	935356	1361128	4075	485276
			98727	840775	899099	3715	465588

Appendix Table 10

BTNW No	Gender	Age	Carmu100	Carmu10	Carmu1	Detergent	Medium
BTNW338	M	72	145155	98513	102152	965	155303
			106971	94584	87352	805	121672
			100075	91693	96619	615	96024
			63954	96196	101214	545	138431
BTNW370	F	66	122053	132101	141227	990	149317
			133051	167781	133738	940	161532
			120720	144320	139518	710	160378
			120125	154001	147142	690	163942
BTNW377	F	45	49040	39536	58038	605	77854
			57566	49501	53779	415	56712
			55757	50104	59886	485	50877
			49180	60499	56330	405	54040
BTNW382	F	75	233	73	103	193	43337
			33	203	42093	143	46950
			203	36856	243	123	47472
			233	143	223	73	52452
BTNW500	F	73	126642	138516	150886	713	206563
			203880	217961	194443	1063	171810
			193611	194149	214035	1303	212774
			194250	230785	194941	1413	200009
BTNW533	M	67	218226	213354	303075	1233	301016
			286618	163339	202376	1423	281332
			302604	119039	203961	933	265587
			257830	115830	157313	873	303710
BTNW544	M	68	163298	167736	159479	350	47710
			131285	114915	139877	400	49156
			101993	151845	133144	460	52590
			97034	119265	108336	240	57773
BTNW546	F	50	73436	102871	120194	1173	156783
			105876	45735	135852	993	178935
			117327	47311	129091	1283	187823
			105412	54701	142676	1313	163396
BTNW590	M	50	113778	139358	113918	1048	107298
			123818	145538	139428	778	126408
			101438	122828	102248	1018	117618
			119988	128508	130118	1448	120438
BTNW597	M	71	54293	56733	65573	753	65103
			41583	57653	74323	573	66923
			45613	74423	81303	683	77783
			58023	66593	79363	343	70023

BTNW365	F	42	1273045	898788	919218	3265	344995
			1302636	1089416	850353	4265	427367
			1535082	1088992	1191591	4775	434793
			1166676	657111	947435	4035	450891
BTNW374	F	34	1016996	1140836	788789	4665	471293
			1209152	798829	741679	4425	443414
			1437931	886668	922794	4075	485276
			1033439	848120	1080043	3715	465588

Appendix Table 11

BTNW No	**Gender**	**Age**	**Temo100**	**Temo10**	**Temo1**	**Detergent**	**Medium**
BTNW338	M	72	110531	91874	91451	965	155303
			108131	95259	90605	805	121672
			94080	110692	97102	615	96024
			57805	68769	88540	545	138431
BTNW370	F	66	101839	144189	147071	990	149317
			120852	158495	152149	940	161532
			106940	149519	140731	710	160378
			114555	161674	140418	690	163942
BTNW377	F	45	51439	59947	47444	605	77854
			61404	54281	53066	415	56712
			70250	54080	44684	485	50877
			56441	49582	50686	405	54040
BTNW382	F	75	233	203	203	193	43337
			27482	26470	34620	143	46950
			26470	43227	12359	123	47472
			243	40136	263	73	52452
BTNW500	F	73	187063	142823	18897	713	206563
			179302	178450	37739	1063	171810
			224168	102798	57031	1303	212774
			161374	66094	47983	1413	200009
BTNW533	M	67	208819	56277	63079	1233	301016
			142156	54921	51035	1423	281332
			136160	80950	171394	933	265587
			106095	73053	40758	873	303710
BTNW544	M	68	129516	138805	175573	350	47710
			204538	123769	161920	400	49156
			131113	150813	181293	460	52590
			132922	143103	159905	240	57773
BTNW546	F	50	75780	126313	95705	1173	156783
			84566	145074	112927	993	178935
			174554	169263	120264	1283	187823
			134790	165463	156803	1313	163396
BTNW590	M	50	119818	119108	120578	1048	107298
			120358	136958	126168	778	126408
			106678	127638	115678	1018	117618
			38568	118948	113398	1448	120438

BTNW597	M	71	52353	61523	46903	753	65103
			58753	72813	66203	573	66923
			60633	84093	71583	683	77783
			64553	79153	81493	343	70023
BTNW365	F	42	360559	983599	854295	3265	344995
			898295	830734	1324120	4265	427367
			191205	1285150	1297188	4775	434793
			240687	1218980	874623	4035	450891
BTNW374	F	34	215277	1386554	1047744	4665	471293
			482566	1309978	789767	4425	443414
			336573	863691	1250950	4075	485276
			208728	873489	641070	3715	465588

Appendix Table 12

			plate T once			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 once	2000	2600	4800	5800	12200	12800
	2000	2200	4600	5400	12000	13200
	2000	2400	4400	6000	12400	13000
	2000	5200	8800	17600	24000	28800
	2000	5200	7800	14400	19200	26200
	2000	4800	7200	14800	15600	26800
	2000	2400	4600	4400	12200	12400
	2000	2200	4000	5800	12800	12800
	2000	2400	4000	5800	13200	13200
IN077 once	2000	7000	9400	9600	17200	17200
	2000	5200	10400	10000	19000	16800
	2000	6400	11000	11000	17400	16000
	2000	2400	3600	2600	5800	7600
	2000	2800	4200	4800	5600	7200
	2000	3000	3600	3400	5600	7000
	2000	2200	2200	3600	5600	11400
	2000	2000	2400	3000	5400	8600
	2000	2600	3000	3200	6200	11400
IN859 once	2000	3200	3200	3200	9200	10200
	2000	2600	3600	2800	8000	9800
	2000	2400	3800	2000	8200	11000
	2000	4000	3800	3600	4200	2400
	2000	2400	3800	4200	3400	2800
	2000	2600	4000	4000	3000	2400
	2000	1400	5600	5000	6200	8000
	2000	3600	4200	4400	3600	6600
	2000	2200	5200	3800	3200	7600
IN1265 once	2000	8200	14600	11200	15000	22800
	2000	6800	13800	14400	15200	21800
	2000	7800	12600	13000	14600	19400

	2000	3400	7000	5000	3200	6800
	2000	2800	4800	6600	4000	7600
	2000	3000	3800	4200	4800	8000
	2000	4000	2200	4800	4200	5800
	2000	3600	2200	5200	5600	5200
	2000	3600	3200	4200	3000	5000
U257 once	2000	14800	55800	27600	40000	16400
	2000	10400	55400	28200	44600	14200
	2000	11400	54400	29800	38200	15200
	2000	5800	3800	13800	14800	8600
	2000	5000	5200	6000	15000	12200
	2000	5000	5600	8200	12200	9600
	2000	8400	21800	12200	12600	8400
	2000	5000	1800	7400	10200	10200
	2000	6800	3000	10800	9200	10800
U373 once	2000	3800	8400	7000	12200	13400
	2000	5600	9400	7600	11600	11200
	2000	4800	8600	9600	12000	11000
	2000	2000	3800	10000	4200	4400
	2000	2000	3800	7800	4400	5400
	2000	3400	3200	8600	5200	5400
	2000	2200	3000	3400	3600	4400
	2000	3600	3600	3600	4400	4800
	2000	4000	4000	4400	5000	5600

Appendix Table 13

			plate T"s" once			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 once	2000	2600	4400	5400	7000	13800
	2000	2200	3400	5400	7600	12800
	2000	2400	3600	5600	7800	13200
	2000	5200	9800	6600	14000	18600
	2000	5200	8200	8400	13000	19600
	2000	4800	9000	9200	13400	22800
	2000	2400	4800	8200	12000	14200
	2000	2200	4400	7200	12000	14800
	2000	2400	4200	7200	12400	15000
IN077 once	2000	7000	8200	10000	15600	17200
	2000	5200	7600	9600	17200	18000
	2000	6400	8800	8800	16600	18200
	2000	2400	3600	4200	5200	9200
	2000	2800	2400	3200	6400	11800
	2000	3000	1800	4400	7600	13600
	2000	2200	3400	3000	7000	4800
	2000	2000	3400	3800	4800	6800
	2000	2600	3400	2400	7000	5600
IN859 once	2000	3200	3000	3200	5400	7800
	2000	2600	2600	2400	5200	6200
	2000	2400	2800	2000	6000	8000
	2000	4000	2800	3400	3000	2600
	2000	2400	2000	4600	3200	2400
	2000	2600	1600	3800	2400	2800
	2000	1400	5000	3000	4200	3000
	2000	3600	3600	4000	2800	2400
	2000	2200	3800	2800	3000	2200
IN1265 once	2000	8200	13000	13800	15600	22400
	2000	6800	14200	16000	15800	25000
	2000	7800	11600	15400	14000	25200
	2000	3400	11000	6000	6800	6200
	2000	2800	4800	5800	5200	5800
	2000	3000	3800	6200	5000	6800
	2000	4000	6400	4600	3200	4200
	2000	3600	5400	5200	5600	4000
	2000	3600	3600	3800	4000	3600
U257 once	2000	14800	21800	23600	40000	24000
	2000	10400	24800	25800	39800	24200
	2000	11400	22000	26400	41000	24400
	2000	5800	16200	17600	14200	10400
	2000	5000	13000	8800	11000	7600
	2000	5000	13200	7600	14600	8000

	2000	8400	26800	8600	10200	8800
	2000	5000	4200	8400	9200	9600
	2000	6800	4000	7600	8800	8800
U373 once	2000	3800	3000	5000	4000	3000
	2000	5600	3400	5400	3600	3000
	2000	4800	3400	4800	3200	2600
	2000	2000	3000	5000	4000	3000
	2000	2000	3400	5400	3600	3000
	2000	3400	3400	4800	3200	2600
	2000	2200	3000	4200	4000	4600
	2000	3600	3400	4400	3800	4200
	2000	4000	3400	2800	4200	5400

Appendix Table 14

			plate cis once			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 once	2000	2600	3400	2400	2200	1600
	2000	2200	2800	2400	2000	1600
	2000	2400	3200	2800	2400	1800
	2000	5200	12800	3000	2000	1200
	2000	5200	11200	2000	1800	1400
	2000	4800	9000	3600	2300	2000
	2000	2400	2600	2000	1800	1200
	2000	2200	3000	1200	1800	1000
	2000	2400	3200	1800	1600	1000
IN077 once	2000	7000	7200	4000	2000	3000
	2000	5200	8600	4600	3200	2800
	2000	6400	8800	3200	2400	2400
	2000	2400	1800	1200	1400	2800
	2000	2800	1400	1400	2200	2000
	2000	3000	1200	1000	1600	2400
	2000	2200	1600	1000	600	3000
	2000	2000	2200	1400	800	2800
	2000	2600	1600	800	1600	2200
IN859 once	2000	3200	3400	2000	2000	2000
	2000	2600	2000	4400	2800	1200
	2000	2400	2000	2800	2200	1000
	2000	4000	3600	3600	2600	600
	2000	2400	2200	2400	3000	800
	2000	2600	3200	3600	2800	800
	2000	1400	2400	2000	1200	800
	2000	3600	2200	1400	1800	600
	2000	2200	2600	1200	1600	600
IN1265 once	2000	8200	7800	7400	5200	6000
	2000	6800	7800	7400	7600	6800
	2000	7800	7400	7200	7600	6600
	2000	3400	10000	5600	1800	1600
	2000	2800	6000	5600	2000	1600
	2000	3000	5800	5200	2600	1000
	2000	4000	5200	2800	5400	4800
	2000	3600	3600	1800	5000	2000
	2000	3600	4800	2000	3400	4200
U257 once	2000	14800	15600	11200	13200	18600
	2000	10400	16800	13200	13200	20400
	2000	11400	15000	14000	13000	17600
	2000	5800	15000	9200	10000	4800
	2000	5000	12200	5400	4200	4000
	2000	5000	11400	4600	4800	5400

	2000	8400	16000	8600	4600	2800
	2000	5000	2800	5800	6000	3200
	2000	6800	3800	7200	4800	4800
U373 once	2000	3800	10600	4800	9600	6000
	2000	5600	8400	4000	11000	5400
	2000	4800	8200	4600	10200	6000
	2000	2000	3800	1600	2200	1200
	2000	2000	4400	4000	3000	1000
	2000	3400	3400	2800	2800	1400
	2000	2200	3200	2800	2000	1200
	2000	3600	3800	2400	1800	1200
	2000	4000	3200	3000	2600	1800

Appendix Table 15

			plate Car once			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 once	2000	2600	4800	5200	12800	23800
	2000	2200	4800	5600	12800	23880
	2000	2400	4600	5600	12400	24020
	2000	5200	4600	6400	19000	22000
	2000	5200	6600	4800	14600	24600
	2000	4800	7200	8400	15600	22800
	2000	2400	3200	4000	13200	25200
	2000	2200	3800	5200	13800	26000
	2000	2400	3200	4800	13600	25800
IN077 once	2000	7000	12800	10200	11000	13000
	2000	5200	11800	12200	9200	12000
	2000	6400	10000	10800	9800	12800
	2000	2400	1200	7400	3600	8400
	2000	2800	1400	5600	5000	7400
	2000	3000	1600	3400	6800	7800
	2000	2200	1800	3200	7400	8000
	2000	2000	1600	2800	5600	9000
	2000	2600	2600	1800	4800	7400
IN859 once	2000	3200	2800	9200	13200	15000
	2000	2600	4400	7800	14000	17000
	2000	2400	3400	8600	12200	18200
	2000	4000	3800	4800	3600	7000
	2000	2400	3800	4800	4600	5400
	2000	2600	4400	4000	3400	4400
	2000	1400	5600	5600	5800	7400
	2000	3600	7200	6000	3600	8200
	2000	2200	7400	5800	4200	7800
IN1265 once	2000	8200	11800	14000	18000	22000
	2000	6800	13800	17800	17800	23800
	2000	7800	12800	15400	19000	21000
	2000	3400	13600	4600	7600	4400
	2000	2800	4400	1200	5800	5000
	2000	3000	6000	2200	5000	6400
	2000	4000	7200	8800	5000	8200
	2000	3600	7800	5200	6000	6600
	2000	3600	7200	5200	5600	6400
U257 once	2000	14800	31800	47400	53000	60200
	2000	10400	28600	46800	55600	59800
	2000	11400	26000	45800	54200	61000
	2000	5800	6000	38000	51600	24600
	2000	5000	5800	27000	53000	23600
	2000	5000	5800	39400	43200	20600

	2000	8400	14800	10000	11000	38600
	2000	5000	4000	9000	11000	30800
	2000	6800	3400	9200	10600	32600
U373 once	2000	3800	8600	7000	13000	15000
	2000	5600	8800	9000	12800	14800
	2000	4800	9200	9000	14000	16000
	2000	2000	1800	4200	5000	4800
	2000	2000	1800	3800	5800	4400
	2000	3400	1000	4000	5800	4000
	2000	2200	3200	4000	5200	6200
	2000	3600	4000	4800	5800	6000
	2000	4000	3000	5000	4800	7000

Appendix Table 16

	Plate Medium		
	Day -4	Day 0	Day 4
1321N1 once	2000	2600	24800
	2000	2200	23600
	2000	2400	24400
	2000	5200	36400
	2000	5200	31200
	2000	4800	29600
	2000	2400	22800
	2000	2200	23200
	2000	2400	24000
IN077 once	2000	7000	15000
	2000	5200	14600
	2000	6400	16400
	2000	2400	7000
	2000	2800	8200
	2000	3000	7000
	2000	2200	7000
	2000	2000	10000
	2000	2600	8200
IN859 once	2000	3200	26600
	2000	2600	28000
	2000	2400	25400
	2000	4000	7200
	2000	2400	9600
	2000	2600	9200
	2000	1400	11800
	2000	3600	10000
	2000	2200	9800
IN1265 once	2000	8200	24400
	2000	6800	25600
	2000	7800	24600
	2000	3400	9400
	2000	2800	8200
	2000	3000	7400
	2000	4000	7600
	2000	3600	9000
	2000	3600	8200
U257 once	2000	14800	64000
	2000	10400	64200
	2000	11400	64200
	2000	5800	24200

	2000	5000	18800
	2000	5000	19600
	2000	8400	40200
	2000	5000	42200
	2000	6800	38400
U373 once	2000	3800	14800
	2000	5600	15200
	2000	4800	15600
	2000	2000	8600
	2000	2000	8400
	2000	3400	7800
	2000	2200	8600
	2000	3600	8800
	2000	4000	9000

Appendix Table 17

			plate T Daily			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 daily	2000	3400	6800	15000	21000	15000
	2000	4800	9000	9000	15200	14400
	2000	6000	8000	7800	17400	16200
	2000	1200	1000	3200	1800	2400
	2000	2400	800	2600	2800	3200
	2000	2000	1800	3200	2200	1800
	2000	2600	2800	6400	3000	3400
	2000	2600	3800	5800	2600	6400
	2000	3200	3200	5600	2200	5000
IN077 daily	2000	7000	3000	4600	5600	8800
	2000	5200	2800	3800	6800	7800
	2000	6400	2400	4600	5400	8000
	2000	2400	2200	4200	5400	7800
	2000	2800	3600	3600	5400	8400
	2000	3000	2400	3600	5400	9200
	2000	2200	5400	5200	7200	10800
	2000	2000	3800	3800	7800	8200
	2000	2600	4400	4400	6000	8800
IN859 daily	2000	3000	2800	5600	9800	10400
	2000	2200	3600	5000	10200	10000
	2000	2600	4000	7200	11000	10800
	2000	5000	3800	3000	1400	2800
	2000	4000	5000	3800	1400	1200
	2000	3400	4600	2600	2200	3000
	2000	2000	5200	4800	2200	2600
	2000	3200	3800	4600	1600	2400
	2000	2400	4200	5400	2400	3000
IN1265 daily	2000	2600	3400	2000	4600	4800
	2000	2200	3600	2800	4800	4200
	2000	3800	4200	3200	5400	6000
	2000	4600	4600	2800	3800	5200
	2000	5200	4200	1000	3800	4800
	2000	5200	3800	2800	4800	4600
	2000	1800	2400	5400	3200	4400
	2000	2200	4200	7800	4600	3600
	2000	3600	2800	6000	5000	5400
U257 daily	2000	6800	19800	15600	22200	21600
	2000	9800	22200	11800	21600	20800
	2000	9600	19600	11000	18000	26800
	2000	5000	13000	6800	8400	13600
	2000	7600	4000	5800	10200	8800
	2000	5200	4000	5400	10400	10800
	2000	5200	3600	3200	10400	14800

	2000	3200	4400	5000	10800	15000
	2000	3600	4000	4400	9600	16200
U373 daily	2000	2000	1600	2600	2800	2600
	2000	2000	1600	2800	3600	2400
	2000	3400	1200	2400	2400	2400
	2000	2200	2600	3000	2600	2200
	2000	3000	2800	3200	2200	1800
	2000	2600	3200	3800	2800	1800
	2000	4000	3000	3400	3000	1800
	2000	2200	2600	4400	3800	1800
	2000	3600	4000	3400	4000	2800

Appendix Table 18

			plate T"s" Daily			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 daily	2000	3400	8800	19200	20000	21600
	2000	4800	10600	11400	15600	21800
	2000	6000	9600	10600	16400	23800
	2000	1200	600	2800	180	2200
	2000	2400	1200	4000	800	2000
	2000	2000	1200	2400	600	2400
	2000	2600	2200	3400	2800	1800
	2000	2600	1600	2000	2400	1600
	2000	3200	2000	2400	2400	1400
IN077 daily	2000	3000	4600	4400	4400	7800
	2000	3600	4000	3200	3800	6800
	2000	3400	3000	4800	3200	8000
	2000	4000	3200	4800	2800	9200
	2000	3400	5800	3200	2400	9600
	2000	3400	4600	5000	5600	8800
	2000	4800	7200	3600	4000	6800
	2000	3000	9200	4200	5000	9200
	2000	2800	8000	5000	7400	9600
IN859 daily	2000	3000	2800	5000	6200	6800
	2000	2200	2600	4800	6800	7200
	2000	2600	3200	5200	7000	8000
	2000	5000	3200	4600	2200	4000
	2000	4000	3400	3600	2400	1600
	2000	3400	2400	3200	2200	3200
	2000	2000	3200	2800	2600	4600
	2000	3200	3200	3000	3200	3600
	2000	2400	3200	3200	2200	4400
IN1265 daily	2000	2600	10200	5400	4000	3800
	2000	2200	2000	10000	3800	3600
	2000	3800	2200	9200	3200	4400
	2000	4600	2400	8800	2600	4800
	2000	5200	1200	7400	1400	4000
	2000	5200	1600	8000	1200	4200
	2000	1800	6600	4000	3600	1200
	2000	2200	5200	4200	5200	2800
	2000	3600	6400	5200	4000	3400
U257 daily	2000	6800	11600	13400	17000	21800
	2000	9800	12600	6800	16600	20800
	2000	9600	10800	4600	14000	20800
	2000	5000	13800	4400	6800	10200
	2000	7600	4400	3800	7200	9600
	2000	5200	1200	4600	8000	11000

	2000	5200	3000	2800	6800	11800
	2000	3200	3200	3000	9000	12200
	2000	3600	3600	2400	7200	13000
U373 daily	2000	2000	1400	2000	2200	2600
	2000	2000	1000	2200	2800	2000
	2000	3400	1400	2800	2600	2600
	2000	2200	2200	3200	3000	2400
	2000	3000	2800	3600	3800	1800
	2000	2600	3400	4200	2800	2400
	2000	4000	2200	3400	3000	1800
	2000	2200	1800	4400	3600	3000
	2000	3600	2200	3200	3400	3600

Appendix Table 19

			plate cis Daily			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 daily	2000	3400	6400	11800	1800	2000
	2000	4800	6400	4400	2400	2400
	2000	6000	6400	2200	1800	1000
	2000	1200	1000	3000	1800	2000
	2000	2400	600	1400	600	1800
	2000	2000	200	1800	1800	1600
	2000	2600	1800	2200	1200	1000
	2000	2600	2000	2400	1200	800
	2000	3200	2800	2400	1200	1600
IN077 daily	2000	3000	5200	5000	1000	800
	2000	3600	4400	4000	600	3000
	2000	3400	3400	5400	1800	1000
	2000	4000	4200	3600	2600	1600
	2000	3400	3400	3200	1200	1800
	2000	3400	3600	3200	2600	1600
	2000	4800	8200	8800	1000	1000
	2000	3000	5400	9000	800	1600
	2000	2800	5800	8200	1200	1400
IN859 daily	2000	3000	3400	2200	2600	1800
	2000	2200	4600	2800	2200	1200
	2000	2600	4000	2200	2400	1400
	2000	5000	2400	2000	4400	1400
	2000	4000	3200	1000	2600	2600
	2000	3400	2400	1200	4000	2200
	2000	2000	4000	7400	2400	2600
	2000	3200	3800	5200	2200	3800
	2000	2400	4400	4400	1400	2600
IN1265 daily	2000	2600	9400	2800	5400	3800
	2000	2200	3800	3800	6000	5000
	2000	3800	2200	3800	4800	4200
	2000	4600	1800	3800	3000	3400
	2000	5200	1200	3400	1800	3000
	2000	5200	2600	3400	2000	2200
	2000	1800	5600	3000	2400	3800
	2000	2200	4800	3600	1800	2000
	2000	3600	4000	2400	3200	1200
U257 daily	2000	6800	10800	10200	8600	4200
	2000	9800	9200	4200	4000	4600
	2000	9600	9000	3400	5000	4600
	2000	5000	18800	6800	4000	3600
	2000	7600	4200	6200	2800	3600
	2000	5200	3400	4800	3000	3400

	2000	5200	1600	2200	3600	4000
	2000	3200	2200	1800	2600	2500
	2000	3600	2000	1800	2400	2200
U373 daily	2000	2000	4400	1600	2200	1200
	2000	2000	3800	4000	3000	1000
	2000	3400	3400	2800	2800	1400
	2000	2200	2800	2400	1800	1600
	2000	3000	2000	2600	1600	1200
	2000	2600	3000	1800	2200	1800
	2000	4000	3200	2400	2000	6000
	2000	2200	3400	2200	1400	4600
	2000	3600	3800	2800	1000	4400

Appendix Table 20

			plate Car Daily			
	Day -4	Day 0	Day 1	Day 2	Day 3	Day 4
1321N1 daily	2000	3400	7400	13000	11200	20000
	2000	4800	7400	7000	14400	21000
	2000	6000	7400	5400	12600	23400
	2000	1200	800	1200	2400	3200
	2000	2400	800	1800	2800	4000
	2000	2000	800	2000	2400	5200
	2000	2600	4200	13200	4600	2600
	2000	2600	4600	12200	3200	4400
	2000	3200	3000	13800	2600	3200
IN077 daily	2000	3000	5000	5000	4800	7000
	2000	3600	4400	4000	6200	8800
	2000	3400	4600	4600	2800	7000
	2000	4000	5600	4200	1200	9000
	2000	3400	4600	5000	1600	8600
	2000	3400	4200	3200	1200	7800
	2000	4800	5400	6400	3800	9200
	2000	3000	5400	3800	3200	6400
	2000	2800	4800	3800	3200	7200
IN859 daily	2000	3000	4000	12000	10800	14000
	2000	2200	4600	9800	12200	15200
	2000	2600	5400	10800	14000	15800
	2000	5000	6000	5800	5600	6200
	2000	4000	5800	6000	7000	4800
	2000	3400	7600	5800	6000	5600
	2000	2000	4400	8000	5000	3600
	2000	3200	5200	5200	4800	4400
	2000	2400	4200	8000	3600	4000
IN1265 daily	2000	2600	10000	5800	5000	7400
	2000	2200	1400	5800	5400	4400
	2000	3800	1600	4600	5000	8000
	2000	4600	2800	4600	5400	15200
	2000	5200	2200	3800	3200	14600
	2000	5200	3800	3600	5200	11000
	2000	1800	2200	5800	9000	6400
	2000	2200	3800	5600	5800	10000
	2000	3600	2000	5200	10200	8800
U257 daily	2000	6800	14800	8400	10200	40200
	2000	9800	14800	3600	11400	40200
	2000	9600	13600	4600	9200	42200
	2000	5000	18000	15000	13200	24800
	2000	7600	3000	15600	12000	31000
	2000	5200	2600	10800	9600	30200

	2000	5200	4600	5800	15200	21000
	2000	3200	4000	8600	16000	19800
	2000	3600	3400	4400	15800	22000
U373 daily	2000	2000	1800	4000	5000	4800
	2000	2000	1800	3800	5800	4400
	2000	3400	1000	4200	5800	4000
	2000	2200	3000	3800	3800	5800
	2000	3000	4000	4600	5200	6000
	2000	2600	4200	5000	4800	6600
	2000	4000	2800	4200	4800	8400
	2000	2200	3000	3800	2800	10400
	2000	3600	4000	3000	3200	7000

Appendix Table 21

			Plate Medium
	Day -4	Day 0	Day 4
1321N1 daily	2000	3400	27400
	2000	4800	29400
	2000	6000	29600
	2000	1200	22800
	2000	2400	22600
	2000	2000	22800
	2000	2600	28200
	2000	2600	29400
	2000	3200	27600
IN077 daily	2000	3000	9800
	2000	3600	8800
	2000	3400	9600
	2000	4000	8400
	2000	3400	8600
	2000	3400	9200
	2000	4800	9600
	2000	3000	11000
	2000	2800	9600
IN859 daily	2000	3000	28000
	2000	2200	26800
	2000	2600	29200
	2000	5000	8600
	2000	4000	6400
	2000	3400	6400
	2000	2000	7200
	2000	3200	7000
	2000	2400	7200
IN1265 daily	2000	2600	7000
	2000	2200	8200
	2000	3800	12600
	2000	4600	10200
	2000	5200	13200
	2000	5200	10800
	2000	1800	15400
	2000	2200	18200
	2000	3600	16800
U257 daily	2000	6800	58800
	2000	9800	57400
	2000	9600	57800
	2000	5000	39800
	2000	7600	42600
	2000	5200	43800

	2000	5200	42000
	2000	3200	40000
	2000	3600	39200
U373 daily	2000	2000	8600
	2000	2000	8400
	2000	3400	7800
	2000	2200	6800
	2000	3000	8000
	2000	2600	7800
	2000	4000	6800
	2000	2200	8200
	2000	3600	6800