Identification of Circulating MicroRNAs as Biomarkers in Glioblastoma

by

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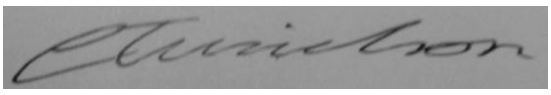
Signature of Candidate 

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Abstract

MicroRNAs (miRNAs) are small RNA sequences 22-25 nucleotides in length which play a role in post-transcriptional gene regulation by binding to target mRNA sequences preventing translation. Changes in miRNA expression can contribute to disease pathogenesis, including gliomagenesis. The release of glioblastoma specific exosomes containing miRNA into the circulation of patients provides a source of biomarkers which could be utilised in a relatively non-invasive diagnostic test. The primary aim of this thesis was to identify secreted biomarkers in biofluids of glioblastoma patients for the diagnosis and prognosis of glioblastoma mulitforme (GBM).

This is the first report of miRNA expression based on the age and sex of GBM patients. GBM and non-cancerous control patients were grouped into age categories (20-39, 40-59 and 60+ years old) and gender. Initial analysis was performed using miScript Brain Cancer Array (n=3 per category) and a total of 28 dysregulated miRNAs were identified in GBM patient serum as candidate biomarkers for further study.

Using a new patient cohort (n=3 per category), further analysis of the 28 miRNAs by qPCR identified five miRNAs with altered expression in GBM serum: miR-34a-5p, miR-92a-3p, miR-20a-5p, miR-30c-5p, and miR-150-5p. Further validation following power analysis identified four of the five miRNA biomarkers: miR-34a-5p, miR-92a-3p, miR-20a-5p and miR-30c-5p to be significantly dysregulated in the serum of GBM patients. Grouping of patients by age and gender identified miR-34a-5p as significantly increased in aged 60+ patients ($p < 0.05$); miR-92a-3p expression was significantly higher in male GBM patients compared to female GBM patients ($p < 0.05$) and miR-20a-5p was significantly higher in a sub group of GBM patients ($p < 0.01$). Moreover, increased expression of miR-20a-5p in the serum of GBM patients was associated with a better median survival compared to those with no change in miR-20a-5p expression.

Investigation into the origin of the serum miRNA biomarkers using qPCR, *in situ* hybridisation and GBM tissue data from The Cancer Genome Atlas (TCGA) identified potential differences in origin of the four miRNA biomarkers; miR-20a-5p in the serum of GBM patients was likely to originate from the GBM. MiR-34a-5p showed increased expression in the GBM tissue analysed by TCGA. In contrast, analysis of matched patient serum and tissue lysate samples using qPCR demonstrated a higher expression of miR-34a-5p in the serum of GBM patients compared to tissue expression, possibly due to increased miRNA secretion by neighbouring non-cancerous cells, or from leukocytes as part of an immune response. Further work utilising larger patient samples could confirm the origin of miR-34a-5p.

Overall four miRNAs were identified in this thesis with altered expression in GBM patients. Further studies could evaluate their use as diagnostic and prognostic serum biomarkers for glioblastoma which could provide a relatively non-invasive alternative to current diagnostic methods requiring surgery.

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Abbreviations

ADAR	Adenosine deaminase acting on RNA
AFP	Alpha-fetoprotein
AGO	Argonaute
Akt	Protein kinase B
AMPK	AMP-activated protein kinase α
ANOVA	Analysis of variance
Anti-DIG-AP	Anti-digoxigenin-alkaline phosphatase conjugate
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
BCL2L11/BIM	Bcl2-like protein 11
BMP7	Bone morphogenic protein 7
BSA	Bovine serum albumin
BTNW	Brain Tumour North West
C. elegans.	*Caenorhabditis elegans*
CAV1	Caveolin 1
CCNE1	Cyclin E1
CDc42	Cell division cycle 42
CDH1	Cadherin 1
CDK4/6	Cyclin-dependent kinase 4/6
CDKN1A/2A	Cyclin-dependent kinase inhibitor 1A/2A
cDNA	Complementary DNA
c-Met	Hepatocyte growth factor receptor
CNS	Central nervous system
CREB	cAMP response element-binding protein
CREB1	cAMP responsive element binding protein 1
CSF	Cerebrospinal fluid
CT	Computerised tomography
Ct	Threshold cycle

CYLD	Cylindromatosis (turban tumour syndrome)
DAB	3,3'-Diaminobenzidine
DALRD3	DALR Anticodon-Binding Domain-containing Protein 3
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
E2F1/2/3/3a	E2F transcription factor 1/2/3/3a
ECACC	European Collection of Cell Cultures
E-cadherin	Epithelial-cadherin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor variant III
EGR1	Early growth response protein 1
EMEM	Eagle's minimal essential medium
EMT	Epithelial-mesenchymal transition
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERK	Extracellular signal-regulated kinase
EZH2	Enhancer of zeste 2 polycomb repressive complex 2
FASTK	Fas-activated serine/threonine kinase
FBS	Foetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FIH	Factor inhibiting HIF
FISH	Fluorescent *in situ* hybridisation
GABRA1	Gamma-aminobutyric acid A receptor, alpha 1
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GTPase	Guanine triphosphatases
HBP1	HMG box-containing protein 1
HER2	Human epidermal growth factor receptor 2
hESC	Human embryonic stem cell
IDH1/2	Isocitrate dehydrogenase 1/2
IGF1R	Insulin-like growth factor 1 recptor

IKBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKKα	IκB Kinase alpha
IKKβ	IκB Kinase beta
IL-1R	Interleukin-1 receptor
IL-1β	Interleukin-1 beta
IL-6R	Interleukin-6 receptor
IRAK1/4	Interleukin-1 receptor-associated kinase 1/4
ISH	*In situ* hybridisation
KCl	Potassium chloride
LASP1	LIM and SH3 protein 1
LKB1	Liver kinase B1
LNA	Locked nucleic acid
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MDM2	Mouse double minute 2 homolog
MGMT	O^6-methylguanine-DNA methyltransferase
MIG6	Mitogen-inducible gene-6
MIR17HG	MiR-17-92 Cluster Host Gene
MiRNA	MicroRNA
MMP	Matrix metalloproteinase
$MnCl_2$	Magnesium chloride
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MVB	Multivesicular bodies
MYD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NBT-BCIP	Nitroblue-tetrazolium – 5-bromo-4-chloro-3-indolyl-phosphate
NEFL	Neurofilament, light polypeptide
NEMO	NF-kappa-B essential modulator
NES	Nuclear export signal
NF1	Neurofibromin 1

NF-κB	Nuclear factor-kappaB
Notch-1/2	Neurogenic locus notch homolog protein -1/2
NRAS	Neuroblastoma RAS viral oncogene homolog
OPTN	Optineurin
p53	Protein 53
PAZ	Piwi, Argonaut and zwille domain
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor, alpha
PDPN	Podoplanin
PI3K	Phosphoinositide 3-kinase
PIWI	P-element-induced wimpy testis
PKM2	Pyruvate kinase, muscle 2
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PTK_7	Protein tyrosine kinase 7
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAN·GTP	Ras-related nuclear protein-guanosine-5’-triphosphate
RB1	Retinoblastoma protein
RBL2	Retinoblastoma-like 2
RECK	Reversion-inducing-cysteine-rich protein
RIP1	Receptor-interacting protein 1
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RND3	Rho family GTPase 3
Robo1	Roundabout homolog 1
ROCK2	Rho-associated protein kinase 2
RT	Reverse transcription
SARA	SMAD anchor for receptor activation

SD	Standard deviation
Smad2/3/4	Mothers against decapentaplegic homolog 2/3/4
snRNA	Small nuclear RNA
SSC	Saline-sodium citrate
SYT1	Synaptotagmin-1
TAB	TGFβ-activated kinase 1 binding protein
TAK1	TGFβ-activated kinase 1
Tap63	Tumour protein p63 isoform
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TGF-βRI/II	Transforming growth factor beta receptor I/II
TIMP3	Tissue inhibitor of metalloproteinases-3
TMZ	Temozolomide
TNF-R1	Tumour necrosis factor receptor 1
TNF-α	Tumour necrosis factor alpha
TNIP1	TNFAIP3-interacting protein 1
TP53	Tumour protein p53
TRADD	Tumour necrosis factor receptor type 1-associated death domain
TRAF2/5/6	Tumour necrosis factor receptor-associated factor 2/5/6
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRBP2	Trans activation responsive RNA binding protein 2
USP15	Ubiquitin carboxyl-terminal hydrolase 15
VEGF	Vascular endothelial growth factor
XCI	X-Chromosome inactivation
YY1	Yin and Yang 1 Protein
ZEB1/2	Zinc finger E-box binding homeobox 1/2
α-SMA	Alpha-smooth muscle actin

1. Introduction

1.1 Glioma

Gliomas are brain tumours that arise from glial cells within the brain, and account for 81% of all malignant brain tumours (Ostrom et al., 2014). The three main types of glial cell are astrocytes, ependymal cells and oligodendrocytes. Tumours that arise from these cell types often share similar characteristics, including indistinct margins and increased malignancy over time (Abrey and Mason, 2009).

Originally, glial cells were believed to have only a supportive function within the brain, but extensive research into the roles of glial cells has revealed that they also play a part in controlling the microenvironment, and the development of the nervous system (Dimou and Götz, 2014). Radial glia are now known to have a role as neural progenitor cells, giving rise to both neurons and astrocytes within the gliovascular network (Dimou and Götz, 2014). In this network, the glia are connected by gap-junctions that allow communication between the cells, providing structural and functional organisation to the brain (Giaume *et al.,* 2010).

Glial cells are also involved in the regulation of synaptic transmission and the formation of new synaptic contacts (Gundersen et al., 2015). It has been proposed that astrocytes may contribute to neurogenesis, originally believed to involve only neuronal cells, by enhancing synaptic activity via the release of glial factors, including cholesterol (Slezak and Pfrieger, 2003) and thrombospondin (Allen and Barres, 2009). The release of cholesterol and thrombospondin contributes to dendrite formation and the promotion of synapse formation respectively, by mediation of cell-cell and cell-matrix interactions (Allen and Barres, 2009).

1.1.1 WHO Classification, Grades of Glioma and Prognosis

The classification of the World Health Organisation grades neoplasms from I-IV, Grade I being a benign tumour with low proliferative potential which, with surgical treatment, can have a promising prognosis and potential cure (Louis et al., 2007). Grade II neoplasms also display low-proliferative potential with infiltration of surrounding tissue, however, grade II tumours have the potential to progress to a higher grade and also present the risk of tumour recurrence following initial

treatment. Grade III tumours often have atypical nuclei and are malignant. Grade IV neoplasms are highly malignant and are often characterised by necrosis and infiltration of surrounding tissue. Even with aggressive treatment, prognosis for grade IV tumours is poor (Ostrom et al., 2014). According to the World Health Organisation's classification of gliomas, ependymomas and oligodendrogliomas are classed as grade II neoplasms. Malignant oligodendrogliomas however, are classed as a grade III (Scheithauer, 2008). Anaplastic astrocytomas are classed as grade III (Kajiwara *et al.,* 2009) and glioblastoma multiforme, the most common form of glioma, is classed as grade IV, due to high malignancy and poor prognosis (Yoon *et al.,* 2010). The grading and characteristics of the most common gliomas types are outlined in Table 1.1.

Table 1.1 Outline of Common Glioma Types, Grades and Characteristics adapted from Louis, 2007

Glioma	Classification	Characteristics
Pilocytic Astrocytoma	Grade I	Benign neoplasm with potential for malignant transformation can be removed by surgical resection.
Ependymoma	Grade II	Slow growing, treatment with surgery and radiotherapy.
Oligodendroglioma	Grade II	Slow growing, survival rate around 11-12 years, treatment with chemotherapy or radiotherapy depending on specific mutation.
Malignant Oligodendroglioma	Grade III	Survival rate around 3-4 years, treatment with chemotherapy or radiotherapy depending on specific mutation.
Anaplastic Astrocytoma	Grade III	Treatment with surgery and radiotherapy often with chemotherapy, survival rate around 2-5 years.
Glioblastoma Multiforme	Grade IV	Poor prognosis, high malignancy, treatment with combined radiotherapy and chemotherapy.

Patients with oligodendroglial tumours have a survival rate of between 4 and 15 years following diagnosis, depending on the grade of neoplasm (Abrey and Mason, 2009). Ependymomas are the least common form of brain tumour and complete surgical resection can often be curative. More malignant cases of ependymomas can be treated with both surgery and chemotherapy, however there is a high risk of recurrence and metastasis (Freyschlag et al., 2011). For low grade astrocytomas, treatment with surgical resection and low dose radiotherapy normally leads to a survival rate between 6-8 years in the majority of cases (Arko *et al.,* 2010).

The prognosis for higher grade gliomas is less ideal, where patients with anaplastic astrocytomas have a survival rate of 2-3 years following surgical and radiotherapy treatment, which may sometimes be combined with chemotherapy (Yoon *et al.,* 2010). The most malignant grade of glioma; glioblastoma multiforme, has the least promising prognosis. Aggressive treatment including maximal surgical resection and chemotherapy is often used in an attempt to prevent recurrence of the tumour after surgery, however, in the majority of cases patient survival in the UK even after aggressive treatment, is only 9 months (Alqallaf et al., 2014).

1.1.2 Epidemiology

In Europe, astrocytic tumours are the most common tumours of the CNS with an incidence rate of 4.8 per 100,000 of the population (Crocetti et al., 2012). Incidence and survival rates for glioma in the United Kingdom and Ireland are outlined in Table 1.2. Between 2000 and 2002, the United Kingdom and Ireland had the highest incidence of astrocytic tumours in Europe with 5.1 cases per 100,000 of the population (Crocetti et al., 2012). The incidence of astrocytic tumours was higher in men than women and highest in individuals over the age of 60 years (Crocetti et al., 2012). Overall in Europe, five-year survival rates were slightly better for women than men; 20.7% compared to 18.7% respectively (Crocetti et al., 2012). Again, the UK and Ireland had the lowest survival rates of Europe at 16.6%, however the poor incidence and survival rates could potentially be due to differences in diagnostic and treatment modalities (Crocetti et al., 2012).

Table 1.2: Incidence per 100,000 people and Survival of Glioma in UK and Ireland between 2000 - 2002, adapted from Crocetti *et al.,* (2012).

Glioma Type	Incidence – Sex		Incidence – Age				Survival (%)	
	Male	Female	0-19	20-39	40-59	60+	1 year	5 years
Astrocytic	5.7	4.0	0.9	2.0	6.2	11.6	31	12
Oligodendroglial	0.4	0.3	0.1	0.3	0.6	0.4	81	51
Ependymal	0.2	0.2	0.2	0.2	0.2	0.2	91	78

1.1.3 Symptoms and Diagnosis

Symptoms of gliomas often consist of seizures caused by the compression of the cortex and headaches caused by intracranial pressure, which can also result in focal neurological deficits, including muscle weakness, numbness, or loss of coordination (Snyder *et al.,* 1993).

The main methods of diagnosing glioma are magnetic resonance imaging (MRI) and histology. MRI provides a detailed anatomical image of the neoplasm and surrounding structures. Histology is most often used to determine tumour grade and characteristics which is used to determine the most effective course of treatment to provide the best prognosis (Abrey and Mason, 2009). In addition, perfusion MRI or magnetic resonance spectroscopy can provide information about tumour vascularity and changes in the normal pattern of biomolecular composition respectively (Abrey and Mason, 2009). For example, a computerized tomography (CT)-MRI scan can be useful when assessing oligodendrogliomas, which often contain deposits of calcium (calcification) and hyperostosis caused by invasion of glioma cells into bone in close proximity to the tumour (Marosi *et al.,* 2008).

1.1.4 Treatment of Glioma

Current chemotherapeutic treatments are outlined in Table 1.3. Initial treatment for glioma entails resection of as much of the tumour as possible depending on the localisation. Functional MRI and diffusion tensor imaging sequences are used to visualise functional areas of the brain and intraoperative cortical and subcortical stimulation are used to monitor essential areas of the brain which must be avoided to

prevent permanent disability of the patient (Preusser et al., 2011). Resection of the tumour is often guided by the use of 5-aminolevulinic acid which is metabolised within the tumour and leads to the accumulation of fluorescent porphyrins. This allows visual identification of tumour margins and enables complete resection (Stummer et al., 2006). Higher grades of glioma however are more difficult to remove surgically due to the infiltrative nature and cancerous cells often remain after surgical resection. Following resection, wafers comprising of the cytotoxic agent carmustine can be implanted. The use of biodegradable carmustine wafers allows localised treatment of the tumour for three weeks (Preusser et al., 2011). Guidelines by the National Institute for Health and Care excellence (NICE) recommend temozolomide (TMZ), an oral alkylating agent, for patients with newly diagnosed high-grade glioma, patients who undergo resection of 90 % of their tumour are recommended to receive carmustine implants.

The main method of treatment for glioma, in particular glioblastoma, is the Stupp regimen which employs TMZ with concurrent radiotherapy followed by TMZ treatment alone (Stupp et al., 2005). TMZ is an alkylating agent which causes DNA mismatches and double strand breaks by alkylation of the O^6-position of guanine. MGMT is capable of repairing the DNA damage caused by TMZ thereby reducing the chemotherapeutic effect of this drug. Hypermethylation of the *MGMT* promoter reduces its ability to repair DNA damage and patients with hypermethylated *MGMT* promoter respond better to treatment with alkylating agents (Von Deimling et al., 2011). *MGMT* status is determined clinically using methylation-specific PCR using primers for methylated and unmethylated CpG areas of the *MGMT* promoter region (Jansen et al., 2010). Patients were found to benefit from improved survival compared to those who received radiotherapy alone (Stupp et al., 2009) and as a result, newly diagnosed glioma patients are often prescribed this course of treatment.

Table 1.3 Current Treatments For Glioma

Drug	Mechanism of action and Limitations	References
Carmustine	Alkylating agent- causes DNA methylation leading to disrupted mismatch repair, senescence and apoptosis.	Papait *et al.,* 2009
Carboplatin	Platinum compound – causes intrastrand and interstrand DNA crosslinks and DNA-protein adducts causing senescence and apoptosis.	Samimi *et al.,* 2004
Cisplatin	Platinum compound – forms DNA-cisplatin adducts leading to senescence and apoptosis.	Samimi *et al.,* 2004
Temozolomide	Alkylating agent- causes DNA methylation leading to disrupted mismatch repair, senescence and apoptosis.	Papait *et al.,* 2009
Radiation	Causes DNA damage, often used in combination with chemotherapeutics such as temozolomide to prevent development of secondary tumours.	Kato, 2010

1.1.5 Molecular Subtypes of Glioblastoma

Genome-wide analysis has revealed glioblastoma to be a heterogeneous group of neoplasms comprising of subtypes which differ in genotypic and molecular alterations and subsequently clinical outcome (Dunn et al., 2012). Analysis of sequencing data from The Cancer Genome Atlas (TCGA) identified four glioblastoma subtypes: classical, mesenchymal, proneural and neural (Verhaak et al., 2010). The genetic aberrations associated with these subtypes are outlined in Table 1.4.

The classical subtype exhibits both a gain of chromosome 7 and a loss of chromosome 10, and for the majority of classical glioblastomas, an *EGFR* amplification. Although mutation of *TP53* is prevalent in glioblastoma, it is not often observed in the classical subtype. The most common mutation of the mesenchymal subtype is *NF1* gene deletion, point mutations or low levels of *NF1* expression often combined with comutation of *PTEN* (Goodenberger and Jenkins, 2012). The neural subtype shares characteristics of the classical and proneural subtypes. Similar to classical glioblastomas, *EGFR* amplification is frequent in the neural subtype. Neural glioblastomas are classified by the expression of neuron marker genes not identified in any of the other subtypes including, *NEFL*, *GABRA1* and *SYT1*. The proneural subtype

exhibits point mutations in *IDH1* and *IDH2* as well as a high level of *PDGFRA* amplification. Proneural glioblastomas which do not display a mutation of *PDGFRA* often have *P13K* mutations (Goodenberger and Jenkins, 2012).

Analysis of clinical characteristics of glioblastoma patients with differing subtypes showed that treatment efficacy of concurrent chemotherapy and radiotherapy was dependent on subtype, where patients with the classical and mesenchymal subtypes showed a reduction in mortality rates in comparison to patients with the proneural subtype in which no difference in survival was seen between treatment regimens. Patients with the neural subtype displayed a slight, but not significant, improvement in mortality (Verhaak et al., 2010).

Table 1.4 Molecular Subtypes of Glioblastoma adapted from Verhaak *et al,* (2010)

Subtype	Aberrant Signalling Pathways	Gene	Type of Mutation
Classical	NES, Notch and Sonic hedgehog	*EGFR*	High level amplification/point mutation/ vIII EGFR mutation
		CDKN2A	Homozygous deletion
		RB1	Homozygous deletion
Mesenchymal	AKT, NF-κB	*NF1*	Hemizygous deletion
Proneural		*PDGFRA*	Focal amplification and high gene expression level
		IDH1	Point mutation
		TP53	Loss of heterozygosity
Neural		*EGFR*	Amplification

1.2 Biomarkers

Biomarkers are defined as objectively measured characteristics within the body which are used to gain information about a particular disease (Pavlou et al., 2013). Due to the poor prognosis of glioma, early detection and effective treatment strategies for glioma patients are vital for improving clinical outcomes, and developing biomarkers for this purpose has long been an aim of research. In addition, biomarkers can provide an insight into the characteristics of the neoplasm. Biomarkers are either produced by the pathological processes of the tumour progression or by the host system in response to the tumour (Manne et al., 2005). As shown in Table 1.5, the information that biomarkers provide about cancer can be used to predict important factors such as prognosis (Lenos et al., 2012, Zougman et al., 2013) and response to therapy (Bauer et al., 2013), as well as to improve diagnosis (Qian et al., 2012) and to assist earlier detection (Manne et al., 2005). Biomarkers can also be used to differentiate between different tumour grades (Ludwig and Weinstein, 2005) and subtypes (Morrison et al., 2012), both of which can be used to tailor treatment strategies.

Current cancer biomarkers in clinical use are outlined in Table 1.5. Although these biomarkers are used for diagnosis and treatment, they lack sufficient sensitivity and specificity required for a successful biomarker (Diamandis, 2010). Current diagnostic biomarkers, such as prostate specific antigen (PSA), are subject to a high incidence of false positive diagnoses (Abu-Asab et al., 2011). Mucin-16, a serum based diagnostic marker of ovarian cancer, lacks sensitivity and specificity for early diagnosis because 50% of patients in the early stages of ovarian cancer do not present with serum mucin-16 expression (Chauhan et al., 2009). Alpha-fetoprotein and beta-human chorionic gonadotropin, both diagnostic markers for testicular cancer, are found to be up-regulated in only 60% of patients therefore risking a false negative diagnosis (Favilla et al., 2010). The up-regulation of HER2 occurs in only 20 to 30% of breast tumours making it an effective biomarker for only a small population of patients (Lam et al., 2013). There is need for biomarkers which can be identified in the majority of a patient population and with a greater sensitivity to reduce the risk of incorrect diagnosis.

Table 1.5 Current cancer biomarkers in use

Biomarker	Cancer Type	Type of Biomarker	Ref
Prostate specific antigen (KLK_3)	Prostate Cancer	Diagnostic	(Abu-Asab et al., 2011)
Mucin-16	Ovarian Cancer	Diagnostic	(Chauhan et al., 2006)
Alpha-fetoprotein (AFP)	Testicular Cancer	Diagnostic	(Favilla et al., 2010)
Beta-human chorionic gonadotropin	Testicular Cancer	Diagnostic	(Favilla et al., 2010)
Her-2	Breast Cancer	Prognostic and risk of recurrence	(Lam et al., 2013)

1.2.1 Current Biomarkers for Glioma

Biomarkers currently used for glioma include O^6-methylguanine-DNA-methyltransferase (*MGMT*) promoter hypermethylation, combined loss of hetrozygosity (LOH) of chromosomes 1p and 19q, LOH of chromosome 10q, mutations of isocitrate dehydrogenases (IDH) and mutations of the epidermal growth factor receptor (EGFR). The main prognostic and predictive biomarker for glioma is MGMT, a DNA repair protein which catalyses the transfer of methyl groups from the O^6-position of guanine to cysteine (Von Deimling et al., 2011). MGMT status is an important factor in the success of chemotherapeutic treatment with alkylating agents such as TMZ (Von Deimling et al., 2011).

Mutations in the *IDH* genes occur mainly in low grade gliomas and secondary glioblastomas and are present in 50-80% of astrocytomas, oligodendrogliomas and oligoastrocytomas (Von Deimling et al., 2011). Mutations in *IDH1* and *IDH2* are rare in primary *de novo* glioblastomas and ependymal tumours and therefore provide a useful method of differential diagnosis (Von Deimling et al., 2011). Patients with *IDH* mutations often have a better prognosis than those without, however *IDH* mutations as prognostic biomarkers are still being defined (Foote et al., 2015). *IDH1* mutations

can be identified using immunohistochemistry with an antibody for mutant Arg132His IDH1 protein (Jansen et al., 2010).

In 60% of glioblastomas the epidermal growth factor receptor (EGFR) is overexpressed and in addition, is often mutated (Jansen et al., 2010). The most common EGFR mutation is EGFR variant III (EGFRvIII). EGFRvIII does not contain a ligand binding domain and as a consequence is constitutively activated causing continual activation of the EGFR-phospho-inositide 3-kinase pathway contributing to oncogenesis. Glioblastomas with the EGFRvIII mutation have been found to exhibit more aggressive characteristics and patients with this mutation may benefit from treatments which target EGFR (Jeuken et al., 2009). EGFRvIII protein can be detected immunohistochemically using antibodies specific for this mutation and amplification of *EGFR* can be determined using fluorescent *in situ* hybridisation (FISH) (Jansen et al., 2010).

For patients with oligodendroglial tumours, co-deletion of the 1p and 19q chromosomal arms is a prognostic biomarker. Patients with this deletion have been found to display enhanced chemosensitivity and longer overall survival which has been attributed to mutations of tumour suppressor genes *FUBP1* and *CIC* mutations in 1p and 19q respectively (Foote et al., 2015). Clinically, 1p/19q status can be determined using FISH or PCR-based LOH assays (Jansen et al., 2010).

The difficulty in identifying a specific biomarker for glioma lies partly in the complex heterogeneous nature of the cancer itself. The multiple mutations a tumour cell undergoes during transformation and the frequency of genomic changes between grades, and sub-types within the grades all contribute to this heterogeneity. Using a group of biomarkers to detect a range of these characteristics or a set of related biomarkers for one specific characteristic is more beneficial than using a single biomarker. This has driven research to the identification of multiple biomarkers which could be used together in a panel (Tainsky, 2009). These panels could be detected using a range of readily accessible high-throughput techniques including qRT-PCR (Garcia-Bilbao et al., 2012, Urquidi et al., 2012) and ELISA (Barderas et al., 2012). One such family of molecules which promises increased sensitivity and specificity as a biomarker is microRNA (miRNA).

1.3 MicroRNAs

1.3.1 MicroRNA Biogenesis and Function

MiRNAs are small non-coding RNAs which are 22-25 nucleotides in length. MiRNAs regulate gene-expression at a post-transcriptional level by binding to mRNA. MiRNA function to regulate cell behaviour (Wang et al., 2011), and modulate gene expression at the post-transcriptional level, by binding to mRNA and suppressing translation (Baraniskin et al., 2012).

Since the discovery of the first miRNA in 1993 (Lee et al., 1993), the biogenesis and maturation of miRNA has been well researched (Graves and Zeng, 2012). To date 2,588 human miRNA genes have been discovered, however the function of many of these has not yet been elucidated. Most miRNA genes are located within introns of both coding and non-coding transcripts, those that are located in the introns of protein-coding genes share the promoter sequence (Ozsolak et al., 2008).

The basic process of miRNA biogenesis involves transcription of the miRNA gene to produce a primary miRNA (pri-miRNA) which is processed into a stem-loop precursor miRNA (pre-miRNA). Cleavage of the pre-miRNA produces a miRNA duplex which is again cleaved into 5p and 3p mature miRNA strands, named in relation to the orientation of the seed sequence. The mature miRNA strand which is not subsequently incorporated into the RISC complex is labelled as miRNA* (Ha and Kim, 2014) (Figure 1.1).

MiRNAs are transcribed by RNA polymerase II producing a long hairpin primary miRNA (pri-miRNA) containing a stem-loop (Lee et al., 2002) (Figure 1.1). The transcription of miRNAs is regulated by transcription factors and epigenetic mechanisms including DNA methylation (Saito et al., 2006) and histone modification (Scott et al., 2006). The stem-loop of the pri-miRNA is cropped by a microprocessor complex, consisting of RNAse III Drosha and DGCR8, to create a smaller hairpin with a 3’ overhang known as a pre-miRNA (Denli et al., 2004).

Pre-miRNA is subsequently exported into the cytoplasm by a transport complex consisting of exportin 5, RAN·GTP and the pre-miRNA. Following transport GTP is hydrolysed, disassembling the complex and releasing the pre-miRNA into the cytosol (Bohnsack et al., 2004). Following translocation into the cytosol, the stem-loop of the

pre-miRNA is cleaved by Dicer which binds to the 3' overhang produced by Drosha within the microprocessor complex, producing a small RNA duplex (Bernstein et al., 2001).

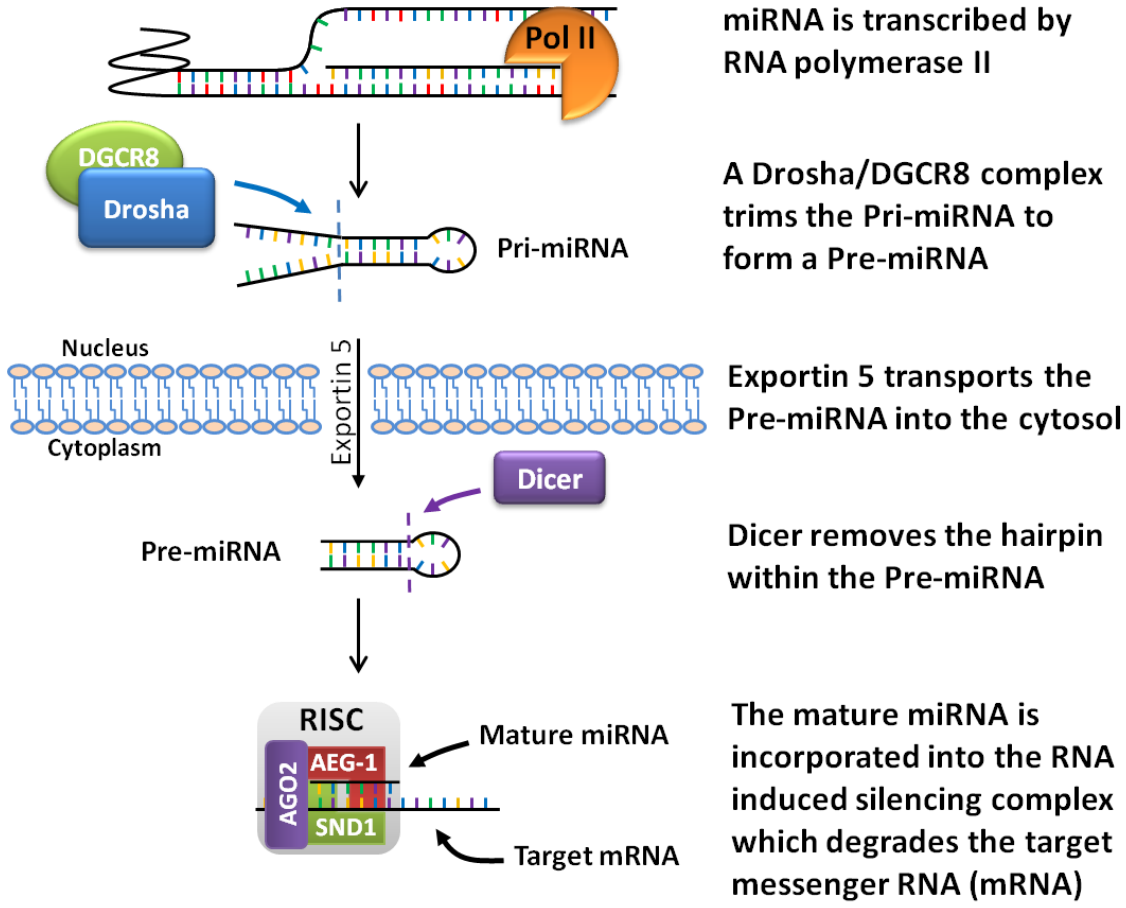


Figure 1.1 MiRNA Biogenesis Pathway. Cleavage and incorporation of miRNA and miRNA* into RISC complex. Pre-miRNA is cleaved by DICER to produce a predominant mature strand and a complementary miRNA* strand, both of which can be incorporated into RISC and function as post-transcriptional regulators (Tumilson, 2015).

The small RNA duplex is loaded onto an AGO protein to form the RNA-induced silencing complex (RISC) (Pratt and MacRae, 2009). The duplex consists of a guide strand and a passenger strand, following loading into RISC the duplex is unwound and removed leaving the guide strand (Kawamata and Tomari, 2010). The 5' monophosphate of the guide strand is anchored to a 5'-phosphate-binding pocket within AGO and the 3' end binds to the PAZ domain of the AGO protein (Schirle and MacRae, 2012). The seed sequence of the guide strand has an A-form helix

conformation, a short compact helical structure with bases not perpendicular to the helix axis, which allows scanning of target mRNA for complementation and cleavage of target mRNA by the PIWI domain (Ha and Kim, 2014).

Mature miRNAs which share 2-8 nucleotides are considered to be from the same family (Bartel, 2009). Some mature miRNAs are encoded by separate loci and are identified by numerical suffixes such as miR-92a-1 and 92a-2. MiRNA genes which share similar sequences are termed 'miRNA sisters', these miRNA may differ by only one or two nucleotides in their seed sequence and share common targets. Sister sequences are identified by lettered suffixes such as miR-181a and 181b. The small difference in seed sequence however, means that these miRNA may also target distinct mRNAs (Ha and Kim, 2014).

1.3.2 MicroRNA Profiles in Glioma

In 2005, the first miRNA profiles were obtained from a range of cancers using bead-based flow cytometry. MiRNA expression profiles were found to differ across cancer types, but a general decrease in miRNA expression was observed in all samples (Lu et al., 2005). One explanation for this could be that exportin 5, necessary for translocation of pre-miRNA into the cytosol during maturation, is truncated in some tumours, and prevents transport of pre-miRNA from the nucleus resulting in a global reduction of mature miRNAs (Ha and Kim, 2014).

Profiling was also able to discriminate between the developmental origin of samples (Lu et al., 2005). This could be particularly useful in metastatic cancers to determine the tissue of origin and location of the primary neoplasm (Rosenfeld et al., 2008). Different mutations within the same cancer type, demonstrated by differences in miRNA expression, were also observed within this study (Lu et al., 2005). These differences in expression have been linked to response to treatment and prognosis (Duffy et al., 2011), and to identify these mutations using specific miRNA profiles would permit better understanding of the tumour and enable improved treatment decisions and patient outcomes (Duffy et al., 2011).

MiRNA signatures have been identified in both glioblastoma tissue and the circulation of glioblastoma patients. Recently, the employment of deep sequencing in which sequences are read multiple times to reduce errors in base calling, produced one of

the largest sets of miRNA profiles for glioblastoma and control brain tissue (Hua et al., 2012b). This study identified 33 up-regulated miRNA in the glioblastoma tissue and 40 down-regulated. In addition, 18 novel miRNA and 16 novel miRNA-3ps were identified (Table 1.6). Moreover, analysis of miRNA isoforms, termed 'isomiRs', identified 23 miRNAs whose most abundant isomiRs were those with 5' variations.

Table 1.6 Novel miRNA-3p identified in glioblastoma and non-cancerous tissue, Hua *et al*. (2012b).

Novel miRNA-3p Discovered	
miR-539	miR-758
miR-382	miR-98
miR-1307	miR-873
miR-212	miR-135a-2
miR-204	miR-511-1
miR-301a	miR-1271
miR-181b-1	miR-381
miR-3676	miR-487a

Increasing the glioblastoma sample size by utilising multiple studies from public databases increases the statistical likelihood that the miRNA signature found will be clinically relevant. One such study from The Cancer Genome Atlas (TCGA) public repository analysed individuals with glioblastoma and identified an expression signature of ten miRNA which could be used to predict survival. Three up-regulated miRNA - 20a, 106a and 17-5p, in the signature were associated with a better survival rate. The remaining seven - 31, 222, 148a, 221, 146b, 200b and 193a, were associated with poor survival. It was concluded that these miRNAs could also be used as therapeutic targets for the treatment of glioblastoma in addition to being used as prognostic markers (Srinivasan et al., 2011). Analysis of the TCGA data showed 19 miRNA in glioblastoma with gender, race, therapy and recurrence dependent differences; including miR-222, 181, 34 and 140. These findings indicated a range of population variables which could affect miRNA expression and therefore biomarker panel selection. Taking these variables into account will increase the success of such panels as well as their specificity towards their intended populations (Delfino et al., 2011).

As previously discussed in Section 1.1.6, glioblastoma consists of subclasses which vary in clinical and genetic characteristics, these subclasses can also be differentiated by miRNA signatures. Analysis of profiles from the TCGA identified five subclasses relating to neural precursor types. Identification of sample subclasses in a study could provide subtype specific miRNA biomarkers. Furthermore, additional information of patient characteristics within the subtypes such as age and response to treatment could be extrapolated to predict survival. MiRNA markers identified for these characteristics included miR-222 for gender, miR-137 as a race dependent marker and miR-140 as a marker for survival (Kim et al., 2011). Another study analysing the TCGA dataset has demonstrated this approach and identified subtype specific prognostic miRNA biomarkers which were subsequently validated using qRT-PCR. Each subtype displayed a panel of biomarkers which contained both positive and negative prognostic markers, some of which were shared by more than one subtype (Li et al., 2014). The classical subtype of GBM displayed seven prognostic miRNAs, five indicative of a poor prognosis – miR-26a, 767-3p, 153, 31, 222, and two indicative of a good prognosis – miR-654 and 422b. Identification of a patient's glioblastoma subtype will provide important information on response to treatment and prognosis as well as providing a diagnosis.

The main appeal of miRNA biomarkers is the tissue and cell specificity of expression. For example, miR-10b is specifically expressed in glioma (Gabriely et al., 2011). MiR-10b has been identified in both low and high grade glioma but is not present in non-cancerous brain tissue. As well as demonstrating tissue specificity in expression profiles, miRNA are known to contribute to the progression of glioma. Through loss-of-function studies, miR-10b was implicated in the regulation of glioma proliferation and apoptosis. In these studies, the overexpression of miR-10b caused the up-regulation of cell cycle regulators including cyclin B1 and D1. Up-regulation of miRNAs usually causes a down-regulation of direct miRNA targets rather than an up-regulation as observed in this study, which led to the suggestion that miR-10b promotes glioma cell growth by indirectly influencing cell cycle regulators (Gabriely et al., 2011) (Figure 1.2). The inhibition of miR-10b led to senescence, growth arrest and apoptosis, both *in vitro* and *in vivo* (Gabriely et al., 2011). The role of miRNA in glioma development and progression, and the tissue specificity, makes miRNA important candidate biomarkers that could provide important characteristic information of a tumour and improve treatment and prognosis.

1.3.3 Role of MicroRNAs in Glioma

Research into the effect of dysregulated miRNA and the role in glioma progression has identified the importance of miRNA within pivotal signalling pathways. These signalling pathways are important when determining glioma characteristics which affect prognosis and treatment outcomes and provide potential targets for biomarker development. Understanding the role of miRNA in signalling pathways and how they affect progression permits the identification of miRNA biomarkers for specific purposes, for example diagnosis or treatment choice. This section outlines the role of miRNA in such pathways which affect glioma behaviour and contribute to a range of characteristics including migration, invasion and proliferation.

Although miRNAs have different targets in different signalling pathways, their effects can all lead to one common tumour characteristic (Figure 1.2). For example, miR-23b, miR-130b and miR-107 regulate different signalling pathways but all contribute to invasion. This underlines the complexity and importance of miRNA in glioma biology and altered expression signatures could again provide important information characteristic of a particular tumour. A recent study into the role of miR-107 in glioma migration and invasion identified Notch2 as a key target (Chen et al., 2013). MiR-107 is down-regulated in glioma, and over-expression in glioma cell lines led to down-regulation of Notch2, which controls a number of tumour characteristics including migration (Figure 1.2). The authors concluded that down-regulation of miR-107 in glioma promotes migration and invasion through Notch2 signalling pathways (Chen et al., 2013). A comparison of miRNA expression profiles in migratory glioblastoma cell lines identified miR-23b as a regulator of both migration and invasion when compared to migration restricted control cell lines (Loftus et al., 2012). MiR-23b down-regulation results in increased expression of the non-receptor tyrosine kinase Pyk2 which leads to cell migration and invasion (Figure 1.2). It was concluded that identifying miRNA important in regulating glioma invasion could provide targets for modulation to reduce invasion and improve treatment outcomes of glioma (Loftus et al., 2012). MiR-23a shares sequence similarities to 23b and has been found to be regulated by cAMP response element-binding protein (CREB) and through increased expression promotes glioma cell growth and survival (Tan et al., 2012b) (Figure 1.2). An isoform of p63 containing a transactivation domain known as TAp63 is known to be structurally and

functionally similar to the tumour suppressor protein p53. TAp63 has been identified as a tumour suppressor which represses metastasis. In a study involving both mouse and human tumour cell lines, Dicer and miR-130b were shown to be targets of TAp63. Binding of TAp63 to the *Dicer* promoter led to transcriptional activation. As well as regulation of Dicer, TAp63 was found to target miR-130b leading to its up-regulation and decrease in invasion (Figure 1.2). Inactivation of TAp63 led to increased metastasis and invasion (Su et al., 2010).

Dysregulation of the transforming growth factor β (TGF-β)/smad pathway in high grade glioma is known to contribute to tumour progression (Song et al., 2012). MiR-182 has been shown as a target of TGF-β which, once activated, down-regulates the expression of NF-κB inhibitors (Figure 1.3). One such inhibitor is ubiquitin carboxyl-terminal hydrolase (CYLD) which is known to be expressed at a lower level in glioma. Although CYLD is down-regulated in glioma, CYLD mRNA levels do not alter, suggesting regulation by direct targeting by miR-182 (Song et al., 2012) (Figure 1.3). NF-κB and the associated signalling pathways are constitutively activated in cancer including glioma, and contribute to tumorigenesis (Karin et al., 2002). The inhibition of the NF-κB negative feedback loop by over-expression of miR-30e-3p constitutively activates this signalling pathway in glioma (Jiang et al., 2012b) (Figure 1.2 and 1.3). Although further research is needed to elucidate the full function of miR-182 in this pathway, miR-182 may also inhibit the negative feedback loop and either miRNA may function individually or together to sustain NF-κB signalling in cancer cells and tumorigenicity in glioma.

To sustain hyperproliferation within a tumour, cells must adapt their metabolic pathways to utilise energy sources available to them both in hypoxic and normoxic conditions. MiR-451 has been found to regulate pathways involved in the adaptation of cancer cells to metabolic stress. MiR-451 is a regulator of the liver kinase B1/Amp activated protein kinase (LKB1/AMPK) signalling pathway. Down regulation of the pathway in the absence of glucose permits the activation of LKB1, allowing cancer cells to adapt to cellular stress by reducing proliferation and activating migratory processes (Godlewski et al., 2010). Furthermore down-regulation of miR-451 in glioblastoma patients is associated with reduced survival, providing a potential prognostic biomarker (Godlewski et al., 2010) (Figure 1.2).

1.3.4 MicroRNA Biogenesis and Glioma

Research is beginning to identify new levels of complexity in miRNA regulation and current studies are showing that deregulation of biogenesis and maturation can also contribute to tumorigenesis. The biogenesis of miRNA is known to be subject to regulation by RNA editing. Pri-miRNA transcripts can undergo conversion of adenosine (A) to inosine (I) by adenosine deaminase acting on RNA (ADAR), known as A to I editing (Kawahara et al., 2008). A to I editing has been found to affect pri-miRNA processing and has been shown in some studies to result in a reduction in levels of mature miRNA (Yang et al., 2006). Alternatively, editing can also lead to the production of mature miRNA with altered sequences, known as 'isomiRs', which target different mRNA transcripts (Kawahara et al., 2008) and can contribute to tumour progression.

Evidence suggests that miRNA transcripts in glioma, particularly high-grade, undergo A to I editing as a result of dysfunctional ADARs (Galeano et al., 2012). Editing within the seed sequence can alter the target of a miRNA. This disrupts regulation of protein expression and contributes to progression (Galeano et al., 2012). On the other hand, lack of editing can also lead to altered gene and protein expression. One such study identified reduced editing of the miR-376 cluster in glioblastoma as a result of lower expression of ADAR and the isoform ADARB1. This caused accumulation of unedited miR-376a-3p transcripts and was shown to contribute to invasiveness and migration of glioma cells (Choudhury et al., 2012) (Figure 1.2). The low expression of ADAR could potentially affect other, currently unknown, targets which may also contribute to the progression of high grade glioma (Choudhury et al., 2012). Another source of alternative miRNA transcripts is the cleavage of miRNA duplexes during maturation. The duplexes are cleaved to produce a functional mature miRNA which is incorporated into the RISC and a miRNA* believed to be degraded following cleavage (Figure 1.1). One such study however, found that miRNA* transcripts are functional and capable of translational repression of mRNA targets, which could therefore have a notable effect in the pathology of disease (Yang et al., 2011).

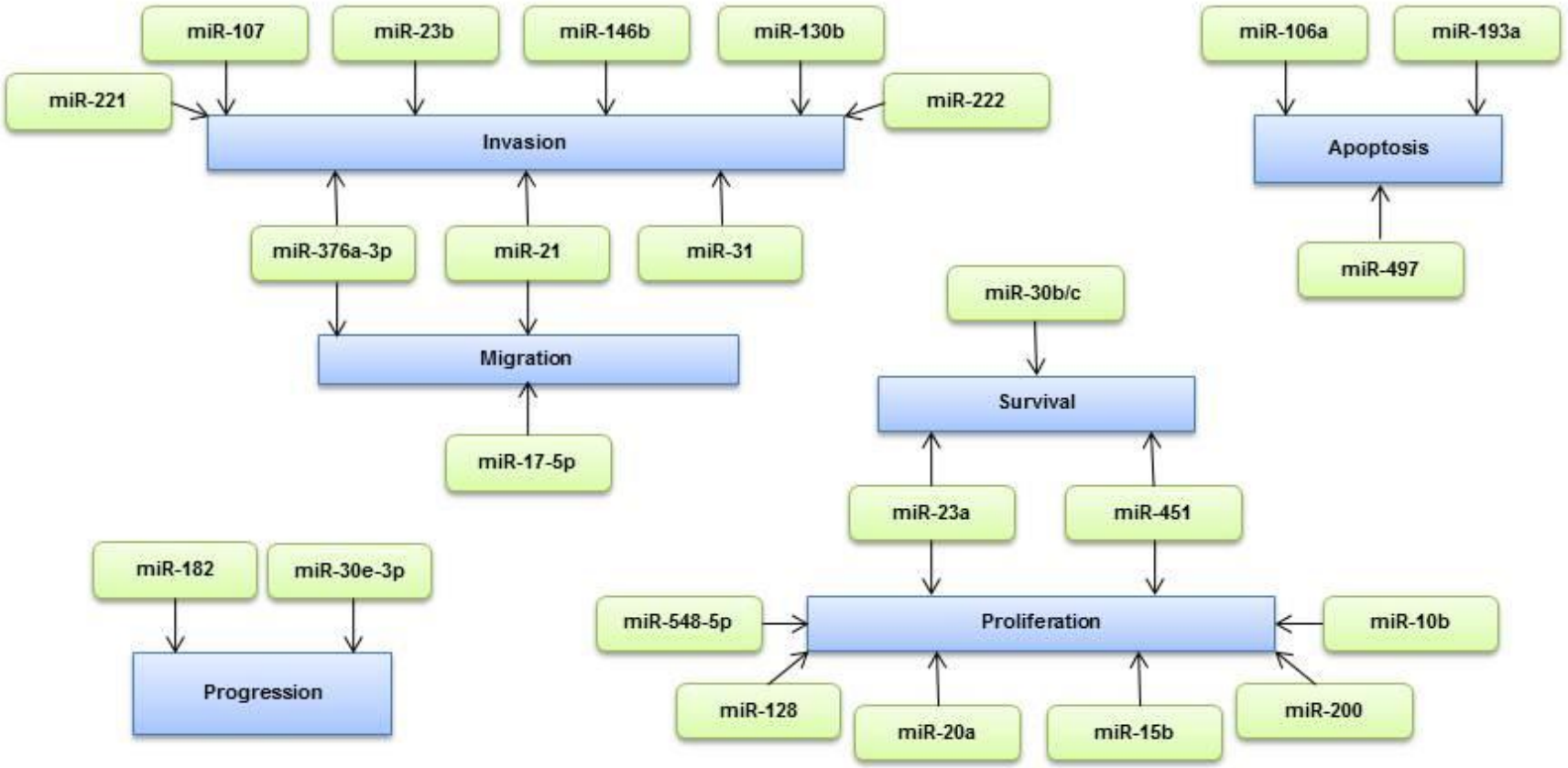


Figure 1.2 Role of miRNAs in Glioma. Altered expression of miRNA affects a number of different targets which influence the same glioma characteristic (Tumilson *et al.,* 2014).

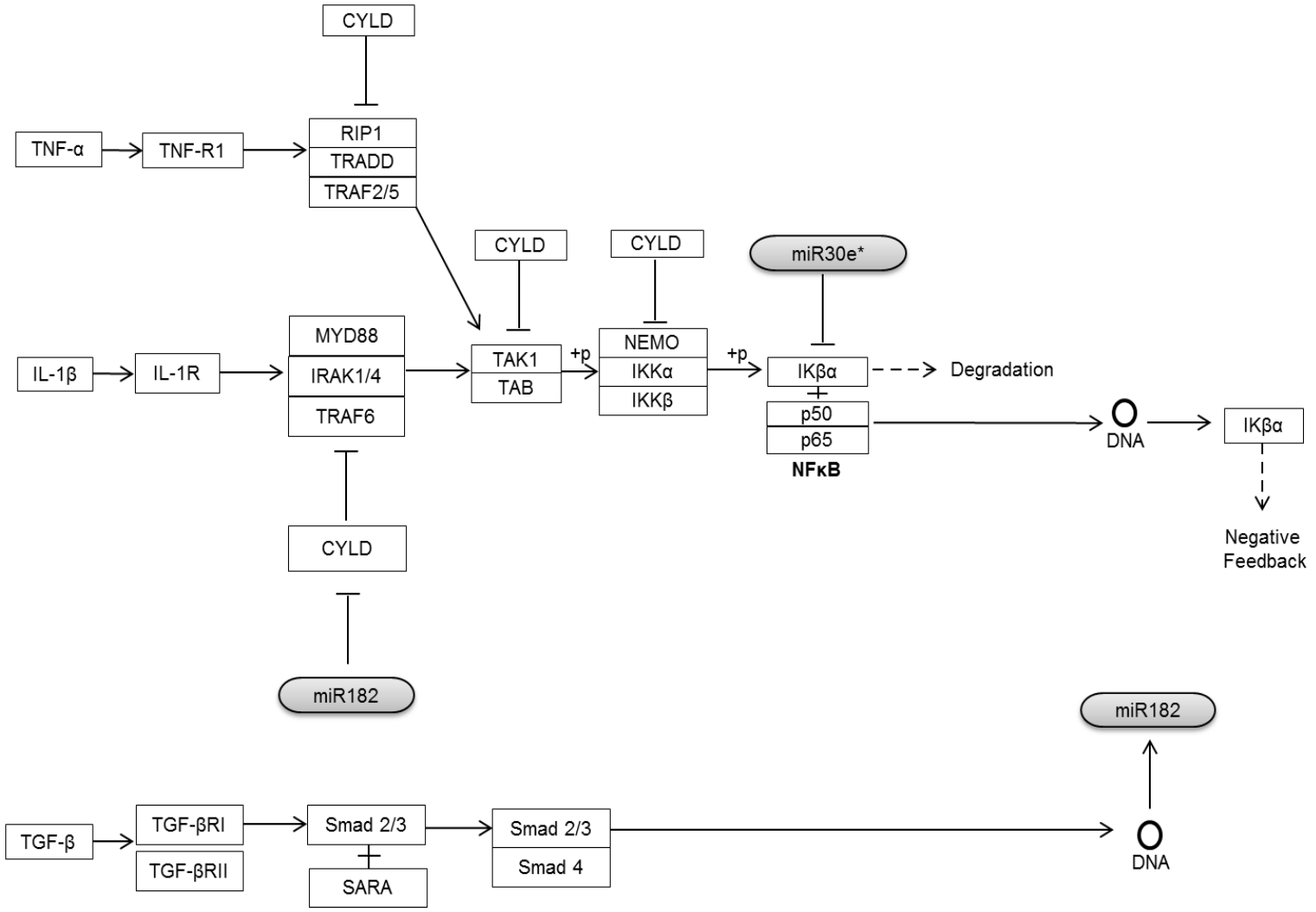


Figure 1.3 Role of miR-30e* and 182 in TGF-β and NFκB signaling contributing to glioma progression adapted from KEGG. Constitutive activation of the TGF-β/Smad pathway increase miR-182 expression. MiR-182 reduces inhibition of NF-κB intermediary signalling molecules by binding to CYLD mRNA causing increased activation of the pathway. MiR-30e* binds to IKβα mRNA preventing action of the negative feedback loop and again, increasing the activity of the NF-κB pathway, and thereby contributing to aggressiveness in glioma (Tumilson *et al.,* 2014).

1.4 Circulating MicroRNA

1.4.1 Exosome Packaging and Release

To prevent degradation in the circulation, miRNA are released by cells in both exosomes and miRNA/protein complexes (Cortez et al., 2011). Exosomes are lipid vesicles ranging between 50-100 nm in size and contain a range of molecules including mRNA, miRNA, DNA and proteins (Hannafon and Ding, 2013). The detection of biomarkers within serum is attractive due to the relatively non-invasive process of collection (Yang et al., 2013a) and subsequent isolation of miRNA from the circulation is a convenient method for biomarker detection.

Exosomes originate from multi-vesicular bodies (MVB) within the cell which fuse with the cell membrane through a secretion pathway involving Rab GTPases to release the exosomes into the extracellular environment (Trajkovic et al., 2008). Analysis of the lipid membranes of exosomes has shown them to be enriched in the sphingolipid ceramide suggesting it to be a pivotal component of exosome formation (Trajkovic et al., 2008). It has been shown that lipid-raft microdomains containing high levels of sphingomyelin promote the inward budding of MVBs following the production of ceramide from sphingomyelin, to form exosomes (Trajkovic et al., 2008). Silencing of two members of the Rab GTPase family, Rab27a and Rab27b, resulted in a reduction of MVB docking to the cell membrane in the HeLa cell line (Ostrowski et al., 2010), showing that Rab27a and Rab27b and the effector proteins can promote intracellular trafficking of MVBs and subsequent exosome secretion in certain instances. Further to this study, the authors investigated the function of Rab27a and Rab27b in *in vivo* murine breast carcinoma models. Although they found that Rab27a modulates exosome secretion as shown in the previous study, Rab27b on the other hand was not required for exosome secretion in the murine models. These findings suggest that MVB trafficking and secretion is a complex pathway which may not be identical across cell types and therefore must be further elucidated (Bobrie et al., 2012).

Analyses of exosome composition have identified a number of different proteins, lipids, mRNAs and miRNAs which differ dependent on the cell type from which they are released and the physiological status of that cell (Hannafon and Ding, 2013). The most commonly identified proteins in exosomes, which seem to be present in most

exosomes regardless of the cell type of origin are membrane transport and fusion proteins, heat shock proteins, GTPases and MVB biogenesis proteins (Hannafon and Ding, 2013). Exosomes frequently contain lipids including cholesterol, sphingolipids and phospholipids (Hannafon and Ding, 2013). Commonly identified exosomal proteins and lipids are outlined in Table1.7.

Table 1.7 Commonly found exosomal proteins and lipids

Proteins	Lipids
Tetraspanins – CD9, CD63, CD81	Cholesterol
Heat Shock proteins – Hspa8, Hsp90	Diglycerides
GTPases – EEF1A1, EEF2	Sphingolipids
MVB biogenesis protein – Alix	Phospolipids
Cytoskeletal proteins – Actin, Syntenin, Moesin	Glycerophospholipids
Metabolic enzymes – GAPDH, LDHA, PGK1, Aldolase, PKM	Polyglycerophospholipids
Signal Transduction proteins – Annexin, 14-3-3ε	
Albumin	

1.4.2 Glioblastoma derived Exosomes

Exosomes are known to be released by both non-cancerous and cancerous cells (Chen et al., 2012) as a form of cellular communication, and perform a variety of functions depending on the contents and cellular context. Exosomes originating from different cell types share a standard set of proteins including tetraspanins and heat shock proteins (Chen et al., 2012), as well as proteins specific to the cell of origin such as the tumour specific EGFRvIII (Skog et al., 2008). The abundance of circulatory exosomes has been shown to increase with tumour progression (Xiao et al., 2012). Although it is unclear how miRNA, mRNA and proteins are packaged into exosomes, research has shown that the packaging of exosomes is a specific and selective process (Chen et al., 2012). Only certain miRNA are incorporated and released into the circulation due to the selectivity of exosome packaging. As a result, certain deregulated miRNA within glioma cells may not be present within isolated exosomes and therefore tissue biomarkers may not translate into circulatory biomarkers. This leads to the conclusion that miRNA signatures for tissue and circulatory biomarkers need to be investigated independently (Jarry et al., 2014).

Analysis of primary glioblastoma tissue and exosomes by qRT-PCR identified the presence of 11 miRNAs, including miR-21 in both sample types. These miRNAs were commonly up-regulated in glioma and present in the exosomes, albeit at a lower level than in the corresponding glioblastoma tissue, however there was a good correlation with the tissue profile leading to the conclusion that circulating exosomes could provide a 'snapshot' of the glioblastoma transcriptome (Skog et al., 2008). Over 28 miRNAs, including miR-21, have been identified in exosomes from the U251 glioblastoma cell line, and at least 22 were significantly enriched in exosomes isolated from culture media compared to cell line expression suggesting specific tumour modulatory roles (Li et al., 2013). Further to this, not only was there an enrichment of certain miRNAs, there was also a higher level of 9 miRNA* including miR-181a*, 93*, 452* and 106a* compared to the mature miRNA form as well as an increase in 3p miRNA when the 5p form was also present. The authors concluded that this change in abundance of the less dominant miRNAs could result in different mRNA being targeted within recipient cells than those in the glioma cells.

Compared to other biomarker types such as protein biomarkers, detection of a small panel of miRNA from the circulation using techniques such as qRT-PCR provides increased sensitivity. The starting concentration of total RNA required from biofluid samples for biomarker detection is relatively low, as little as 25 pg of RNA (Chen et al., 2005). The selective packaging of miRNA into exosomes containing components indicating the cell of origin and the detection of tissue specific miRNAs in the circulation provides specificity for miRNA biomarkers. In addition the detection of more than one tissue specific miRNA provides the advantage of reducing overlap with other pathologies which may share deregulated miRNA biomarkers (Sheinerman and Umansky, 2013).

Solexa sequencing of pooled sera identified a panel of seven down-regulated circulating miRNA which could be used as a signature for glioma (Yang et al., 2013a). The seven miRNAs which made up this panel included miR-15b*, miR-23a, miR-133a, miR-150*, miR-197, miR-497 and miR-548b-5p. In addition to this main panel, certain miRNA made up smaller groups which could be used to differentiate between benign and malignant astrocytomas, as well as other primary brain tumours (Yang et al., 2013a). The expression of miR-15b*, miR-23a, miR-150*, miR-197, and miR-548b-5p

were significantly up-regulated in malignant neoplasms in comparison to benign samples.

A less studied source of miRNA biomarkers is plasma, one such study showed the levels of certain miRNA including 21, 128 and 342-3p, were altered in the plasma of glioblastoma patients in comparison to non-cancerous plasma samples (Wang. et al., 2012). This particular study used qRT-PCR to identify target miRNAs from individual plasma samples which could account for differences between the miRNAs identified in the seven miRNA panel, as well as other variables including a smaller sample size, different extraction and qRT-PCR reagents being used. This study not only identified potential miRNA biomarkers but also showed that isolation of miRNA need not be restricted to serum alone.

Due to the close proximity of cerebrospinal fluid (CSF) to the brain and spinal cord, disorders arising in the CNS can often cause an alteration in CSF composition. The presence of a glioma within the CNS results in the alteration of CSF composition as a result of: humoral responses (Tainsky, 2009); breakdown of structures within the CSF such as the blood-brain barrier (BBB) (de Bont et al., 2006); or as a result of up-regulated production and secretion by the glioma cells themselves (Niclou et al., 2010). Alternatively, the function of the structures related to CSF production and composition can become affected and subsequently contribute to pathophysiology (Tainsky, 2009). The role of CSF in the pathogenesis of glioma is mainly the delivery of substances that play a role in tumourigenesis. These substances include growth factors, hormones and signalling molecules, as well as many other components of CSF and are believed to contribute to a number of glioma characteristics such as invasion, migration and metastases (Tainsky, 2009).

The presence of miRNA in the CSF of glioma patients has initiated studies into the potential of CSF as a source of biomarkers. Although CSF is not routinely obtained from patients with glioma (Baraniskin et al., 2012), the proximity of CSF to a glioma, and its isolation from general circulation, means it could provide a more specific and accurate miRNA profile in comparison to serum and plasma (Teplyuk et al., 2012). In 2012, the identification of miR-21 and 15b within the CSF of patients with malignant glioma was reported (Teplyuk et al., 2012). MiR-21 promotes migration and invasion by targeting MMP inhibitors RECK and TIMP3 (Gabriely et al., 2008), miR-15b is a cell cycle

regulator which targets CCNE1 (Xia et al., 2009) (Figure 1.2). This initial study highlighted CSF as a potential source for miRNA biomarkers and concluded that in the future, CSF miRNA could differentiate between glioma subtypes (Baraniskin et al., 2012). Following this, a pilot study (Teplyuk et al., 2012) identified miRNA which could potentially be used to diagnose glioblastoma or discriminate between glioblastoma and metastatic cancer, once again highlighting the potential of cerebrospinal fluid as a source of biomarkers for glioma. Both studies highlighted miR-21 in the CSF of glioblastoma patients but the latter study also found miR-10b significantly up-regulated. Furthermore, members of the miR-200 family, which share the same seed sequence to each other, were up-regulated in the CSF of patients with brain metastasis. A member of the miR-200 family, miR-200b targeted CREB1 and regulated glioma growth thereby acting as a tumour suppressor (Figure 1.2) (Peng et al., 2013). Table 1.8 outlines deregulated miRNAs in glioma, sample types they have been identified in and the target signalling pathways.

Table 1.8 Deregulated microRNAs from different sample types in glioma and their corresponding signalling pathway

MicroRNA	Up/Down regulation	Targets/Signalling Pathway	Sample Type	References
10b	Up	BCL2L11/Bim, CDKN1A/p21, CDKN2A/p16	Tissue, cell lines, CSF	(Gabriely et al., 2011, Teplyuk et al., 2012)
15b*	Down	-	Serum	(Yang et al., 2013a)
15b	Up	CCNE1	CSF	(Baraniskin et al., 2012)
17-5p	Up	Cyclin D1	Tissue	(Srinivasan et al., 2011)
20a	Up	E2F1, Cyclin D1	Tissue	(Srinivasan et al., 2011)
21	Up	PI3k/Akt	Tissue, serum, plasma, CSF	(Baraniskin et al., 2012)
23b	Down	Pyk2	Cell lines	(Loftus et al., 2012)
23a	Down	IL6R	Serum	(Yang et al., 2013a)
30e-3p	Up	NF-κB	Tissue, cell lines, primary culture	(Jiang et al., 2012b)
31	Down	FIH	Tissue	(Srinivasan et al., 2011)
106a	Up	FASTK	Tissue	(Srinivasan et al., 2011)
107	Down	Notch2	Tissue, cell lines	(Chen et al., 2013)
128	Down	E2F3a	Plasma	(Wang. et al., 2012)

133a	Down	CAV1, LIM, LASP1	Serum	(Yang et al., 2013a)
146b	Down	MMP	Tissue	(Srinivasan et al., 2011)
148a	Down	DNMT1	Tissue	(Srinivasan et al., 2011)
150*	Down	-	Serum	(Yang et al., 2013a)
182	Up	TGFβ/smad, NF-κB	Tissue, cell lines, primary culture	(Song et al., 2012)
193a	Down	Mcl-1	Tissue	(Srinivasan et al., 2011)
197	Down	Fus1	Serum	(Yang et al., 2013a)
200b	Down	RND3	Tissue	(Srinivasan et al., 2011)
221	Down	PTEN, p27 and p57	Tissue	(Srinivasan et al., 2011)
222	Down	PTEN, p27 and p57	Tissue	(Srinivasan et al., 2011)
342-3p	Down	BMP7	Plasma	(Wang. et al., 2012)
451	Down	LKB1/AMPK	Cell lines	(Godlewski et al., 2010)
497	Down	BCL2	Serum	(Yang et al., 2013a)
548b-5p	Down	PTEN, CDK6	Serum	(Yang et al., 2013a)

1.4.3 Uptake of Exosomes by Recipient Cells

Tumour derived exosomes have the ability to transfer their contents to recipient cells and promote angiogenesis and metastasis. It is believed that there may be a number of mechanisms of exosome uptake, which could be dependent on the target cell, however this remains to be elucidated (Svensson et al., 2013). Recently, uptake of exosomes originating from GBM cells has been shown to occur by non-clathrin dependent, lipid raft-mediated endocytosis and ERK1/2-HSP27 signalling (Svensson et al., 2013). Further to this, heparan sulfate proteoglycans (HSPGs) have been shown to play a role in mediating the uptake of exosomes by recipient cells. The binding of exosomes to HSPGs induces activation of the ERK1/2 signalling and subsequent uptake (Christianson et al., 2013). Malignant tumours are often characterised by an acidic microenvironment and it has been suggested that the low pH of the tumour microenvironment may promote both the release and uptake of exosomes (Parolini et al., 2009). An acidic microenvironment influences membrane rigidity of exosomes and increases fusion capacity with recipient cells. The increased fusion capacity of exosomes in a low pH is believed to be a result of the increased formation of lipid rafts in the exosome membrane, which modulate the efficiency of membrane fusion (Parolini et al., 2009). The increased ability of exosomes to fuse with recipient cells in an acidic environment suggests that exosome trafficking occurs more frequently within tumours compared to non-cancerous tissues (Parolini et al., 2009).

1.5 MicroRNAs as Biomarkers

1.5.1 MicroRNA Biomarkers for Diagnosis and Prognosis

The diagnosis of glioma is currently performed by MRI and subsequent histological examination of tumour sections which are obtained by surgery. Therefore the development of a non-invasive test for glioma diagnosis would be highly beneficial to patients and would potentially allow early diagnosis. Circulating miRNA could provide a minimally invasive diagnostic tool however the main focus of research has been on miRNAs for the prediction of prognosis and response to therapy. One study which investigated circulating miRNAs for the diagnosis of glioma was Manterola *et. al.* (2014) who identified a signature comprising of two miRNA; miR-320 and miR-574-3p,

and one small non-coding RNA, RNU6-1 which were significantly associated with GBM diagnosis. This signature was identified by the isolation of microvesicles from the serum of patients and subsequent profiling using qRT-PCR and the signature was validated using a second independent cohort of patients (Manterola et al., 2014). MiR-125b has also been identified as a potential diagnostic biomarker for glioma, analysis of serum samples obtained from patients using qRT-PCR identified down-regulation of miR-125b compared to control samples (Wei et al., 2014). Analysis of the diagnostic accuracy of miR-125b identified a sensitivity of 82 % for detection of glioma grades II-IV (Wei et al., 2014).

Evaluation of the miR-29 family, miR-29a, miR-29b and miR-29c, in the serum of glioma patients identified that this family of miRNAs had good sensitivity and specificity for the diagnosis of high-grade glioma (Wu et al., 2014). Serum samples from high-grade and low-grade glioma patients were analysed using qRT-PCR and the miR-29 family was found to be down-regulated in both high and low-grade glioma serum, with a greater down-regulation observed in high-grade samples (Wu et al., 2014). The authors concluded however, that the miR-29 family may not have sufficient sensitivity to detect early-stage glioma but could be useful in the identification of glioma progression (Wu et al., 2014).

A number of studies have identified miRNAs within tumour tissue for predicting the prognosis of glioma patients. Some miRNAs identified as prognostic biomarkers are outlined in Table 1.9. One such study identified miRNA expression patterns which could be used to identify subgroups of glioblastoma patients with differing prognosis (Niyazi et al., 2011). Tissue sections obtained from glioblastoma patients were analysed using microarray and identified two differing expression patterns of 30 miRNA which was used to group patients into ‘long-term’ or ‘short-term’ survivors (Niyazi et al., 2011). This study, however was limited by the small sample size of patients and was retrospectively performed.

Analysis of serum from astrocytoma patients identified three serum miRNAs; miR-19a-3p, miR-106a-5p and miR-181b-5p, which were significantly correlated with survival (Zhi et al., 2014). Patients with an up-regulation of these three miRNAs exhibited a poorer survival rate than those with a down-regulation (Zhi et al., 2014).

Table 1.9 Prognostic miRNAs and miRNA signatures identified in serum and tissue samples from glioblastoma.

MiRNA	Source	Expression	Prognosis	Reference
miR-124	Frozen tissue	Down-regulation	Poor	(Chen et al., 2015)
miR-155	Frozen tissue	Up-regulation	Poor	(Sun et al., 2014)
30 miRNA signature	FFPE tissue sections	Up and down-regulation	Long-term and short-term survival	(Niyazi et al., 2011)
miR-335	Frozen tissue	Up-regulation	Poor	(Jiang et al., 2012a)
miR-196a, miR-196b	Frozen tissue	Up-regulation	Poor	(Guan et al., 2010)
Ten miRNA signature	TCGA dataset	Seven up-regulated, three down-regulated	Good and poor prognosis	(Srinivasan et al., 2011)
Six miRNA signature	FFPE tissue	Up and down-regulation	Good and poor prognosis	(Sana et al., 2014)
Six miRNA signature	Frozen tissue	Up and down-regulation	Good and poor prognosis	(Barbano et al., 2014)
miR-19a-3p, miR-106a-5p, miR-181b-5p	Serum	Up-regulation	Poor Prognosis	(Zhi et al., 2014)

1.5.3 MicroRNA biomarkers for response to therapy

In the treatment of glioma, chemoresistance can be a pivotal factor in the prognosis of a patient. The ability to predict response to treatment could improve prognosis by selecting the right treatment course as soon after diagnosis as possible and permit rapid adaption of treatment to the acquisition of chemotherapeutic and radio-resistance. This not only benefits the patient by improving their prognosis but also improves the cost-effectiveness of chemotherapeutics by using them only when they are expected to succeed. Furthermore, the use of predictive biomarkers in clinical trials could identify patients most likely to respond to new anti-cancer therapies, thereby accelerating the development of novel therapeutics (Carden et al., 2010). Currently, the gold standard for glioblastoma treatment is TMZ usually combined with

radiotherapy. Only a small subset of patients respond to TMZ treatment, as patients with the functional O^6-methyl guanine methyltransferase (MGMT) DNA repair protein reverse the guanine methylation caused by TMZ leading to chemoresistance and limited success of this drug (Zhang et al., 2012). MiR-181d could be used as a biomarker to identify patients who would respond the best to TMZ, because MGMT is a candidate target of miR-181d, and a higher expression of miR-181d correlates with a lower expression of MGMT and subsequently improved response to TMZ (Zhang et al., 2012).

Whilst miR-181d up-regulation may correspond to a better response to TMZ, up-regulation of miR-21 on the other hand, may predict poor response linked to the high rate of TMZ resistance which develops in patients. MiR-21 is one of the most frequently up-regulated miRNAs in glioblastoma and has been found to protect U87MG glioblastoma cells from TMZ-induced apoptosis (Shi et al., 2010). Inhibition of miR-21 in the resistant D54MG cell line enhanced chemosensitivity to TMZ (Wong et al., 2012). Both of these findings suggest that miR-21 could be used as a biomarker to predict or monitor the acquisition TMZ resistance in glioblastoma patients to enable quick adaptation in treatment strategy and maintain a good prognosis. Further to the role as a chemotherapeutic marker, miR-21 has also been shown to function in the acquisition of radio-resistance. Analysis of radio-resistance in a number of glioblastoma cell lines including U87MG and U373 showed that radio-sensitivity was closely related to the expression level of miR-21 (Gwak et al., 2012). The silencing of miR-21, using anti-miR-21 in radio-resistant malignant glioma cell lines led to the sensitisation of these cells to radiation (Gwak et al., 2012). Anti-miR-21 was found to sensitise U87MG and U373 cells through inactivation of the PI3K/Akt signalling pathway. Whilst these findings point towards a mechanism of acquired radio-resistance, they also highlight miR-21 levels could be an important predictor of acquired radio-resistance which if monitored would permit quick adaptation of treatment plans and effective treatment of the glioblastoma as it progresses.

As well as affecting TMZ and radio-resistance miRNA 21 along with miR-30b and 30c have been identified as regulators of TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. These three miRNAs therefore, could affect the sensitivity of glioma cells to treatment with the TRAIL ligand (Quintavalle et al., 2012). A study of glioma cell

lines and primary cultures found that miRNA-21, 30b and 30c were significantly up-regulated in TRAIL-resistant glioma cell lines (Quintavalle et al., 2012). Consequently TRAIL-sensitive cells were found to exhibit down-regulation of these three miRNA. The targets of these miRNA were found to include caspase-3 by the miR-30 family and Tap63 by miR-21. It was concluded that these miRNA regulate apoptotic programs within glioma cell lines. The results obtained also demonstrated a difference in miRNA expression between TRAIL sensitive and resistant cells (Quintavalle et al., 2012). These miRNA biomarkers for response to treatment and therapeutic resistance are outlined in Table 1.10.

Table 1.10 MicroRNA Biomarkers for Response to Therapy

MiRNA	Therapeutic Response
MiR-181d	Temozolomide resistance
MiR-21	Temozolomide resistance, TRAIL resistance, Radio-resistance
MiR-30b	TRAIL resistance
MiR-30c	TRAIL resistance
MiR-425-5p	Radiochemotherapy response
MiR-93-5p	Radiochemotherapy response

1.6 Challenges in biomarker discovery

Although miRNA appear to be extremely promising biomarkers and research into the identification of miRNA biomarkers for cancer remains on the increase, to date, there are no clinically utilised miRNA biomarkers for glioma. On-going clinical studies investigating miRNA profiles for a number of cancers have been outlined in a previous review (Nana-Sinkam and Croce, 2013). Researchers believe that the lack of clinical miRNA biomarkers compared to the number identified in research, is due to limitations in standardising of sample type collection (Git et al., 2010), determining optimal

methods of extraction, and processing of both samples (McDonald et al., 2011) and data (Qureshi and Sacan, 2013), all of which can affect the reproducibility of individual findings.

The choice of sample type, and the origin of miRNA, can be a limitation in biomarker discovery. An analysis of circulating miRNA isolated from plasma samples identified miRNA of hematopoietic cell origin. The presence of these miRNA in plasma samples is a pre-analytical variable which could affect the analysis of circulating miRNA expression. The employment of sub-fractionation to remove cellular miRNA improves specificity of circulating miRNA markers. The use of miRNA categories for the classification of circulating and cellular miRNA in plasma samples was proposed as a method that could improve miRNA biomarker studies (Duttagupta et al., 2011). The expression of specific miRNAs can differ between sample types used in studies (Wang. et al., 2012). Whilst miR-15b did not appear to be significantly dysregulated in a study using plasma samples, a significant increase in miR-15b levels in CSF samples has been reported (Baraniskin et al., 2012).

Initial collection of sample biofluids must also be taken into account as there are variables in this process that may also affect miRNA data. Differences in collection tube type and phlebotomy techniques have both been suggested as factors which may cause variability in circulating miRNA expression. Hemolysis of the sample can increase the abundance of certain miRNA in biofluid samples which can subsequently affect biomarker selection (McDonald et al., 2011) therefore effective removal of cells is essential when using plasma and serum samples for biomarker detection. In addition to cells, other components of serum and plasma which are present in high levels, such as lipids and proteins, can affect the isolation of the RNA (Zampetaki and Mayr, 2012).

Although tumour cells are known to shed exosomes, non-neoplastic cells and platelets also release exosomes. Serum isolated exosomes were used to identify mRNA expression patterns in glioblastoma patients (Noerholm et al., 2012). When compared with control serum samples, the expression patterns of mRNA within the exosomes could differentiate between those patients with glioblastoma and those without, however, the down-regulation of mRNA in serum exosomes could either be due to tumour exosomes, or from exosomes with an altered expression due to disease states and lifestyle from non-cancerous cells (Noerholm et al., 2012). Like mRNA expression,

the release of miRNAs from non-neoplastic cells must be taken into account in circulating miRNA expression studies for the identification of biomarkers (Pritchard et al., 2012b).

The tissue and cell specificity of miRNA and individual variations due to diet, disease, infection or even age and gender, could potentially affect the detection of circulating miRNA biomarkers. One such review illustrated the influence of a number of environmental factors including diet, infection and stress on epigenetic mechanisms including microRNA expression (Mathers et al., 2010). Although the focus was the effect of these factors on cancer susceptibility, it also shows that inter-individual variations in lifestyle can affect the expression of miRNA which could subsequently affect the detection and use of miRNAs as biomarkers. The analysis of gene expression in the peripheral blood of healthy subjects also demonstrated inter-individual variability depending on the ratio of different blood subsets, age, gender and even the time of day when the blood sample was collected (Whitney et al., 2003). This has an important meaning for the identification of circulating biomarkers from blood samples as inter-variation in both cancerous and control sample sets may alter the miRNA levels within samples. Subsequently selection of a miRNA biomarker deregulated as a result of the glioblastoma and not of any other factor becomes difficult and accurate patient information capturing this data needs to be recorded in public miRNA databases.

The presence of other diseases or infections, which is likely in older patients, could also skew data toward deregulated miRNAs as a result of an unrelated pathology leading to the failure of that miRNA biomarker in further studies. MiRNA expression and levels are also subject to change following therapeutic treatment (Summerer et al., 2013). MiR-425-5p and 93-5p abundance alters in response to radiochemotherapy in head and neck squamous cell carcinoma patients and could be used as a biomarker to monitor response to treatment. At the same time this study illustrates the importance of knowing the origin of samples used in studies and any treatment undertaken before collection of the sample. Ideally, samples for use in biomarker identification studies, particularly for diagnostic markers, should be taken before any treatment or surgery to ensure any candidate miRNAs are up-regulated as a result of the glioblastoma and not the response of the tumour to treatment. In addition, this further highlights the need

to analyse data subsets where patterns of miRNA profiles can be extrapolated from patients grouped depending on age, gender, sampling time, treatment and other variables.

The methods of isolating and measuring miRNA expression employed in a study can also affect the identification of miRNA biomarkers and cause variability between studies. A comparison of two RNA isolation methods and their effect on serum microarray expression analysis identified differences in miRNA expression between the two methods. Overall, a higher expression of miRNA was observed when using a total RNA isolation method (guanidine isothiocyanate) compared to a silica-gel column based method for isolating small RNA alone (Podolska et al., 2011). It was concluded in the latter that the high lipid content of serum samples affected the isolation of RNA. The total RNA isolation method removes lipids from the sample before isolation and therefore provides a better recovery of RNA (Podolska et al., 2011). Following isolation, miRNA expression is frequently determined using microarray and qRT-PCR methods. The data produced from these methods requires an endogenous candidate ubiquitously expressed across all samples for normalization (Peltier and Latham, 2008). For serum and plasma samples, there have been no significant endogenous control established for use in normalisation (Mitchell et al., 2008). As a result, researchers use a number of different methods such as mathematical models or synthetic miRNA spike-ins, to normalise their data. While this permits research into circulating miRNA to be performed, the resulting data from different studies is often incomparable and not reproducible contributing to the failure of these biomarkers to reach clinical trials. Standardisation of methods for extraction and data analysis would therefore improve the reproducibility of biomarker research and speed the discovery of a clinically effective biomarker panel.

There is a need for standardisation of isolation and analysis techniques to improve the reliability of candidate miRNA biomarker data. A large scale multi-centre study into the optimal techniques and protocols for miRNA biomarker discovery from a range of sample types including tissues and biofluids would provide data which could be used to reach a consensus on standard techniques to be used by all researchers. This would subsequently improve the success of miRNA biomarkers for all disease states including glioma. Conducting a study to identify miRNA biomarkers from biofluids must be

strictly regulated throughout the whole process from patient selection and sample collection through to processing and analysis. Stringent guidelines to minimise variability in patient cohorts, and validate the source of miRNAs, normalisation methods and good experimental design should be agreed upon to ensure reproducibility of data and the efficacy of these markers during further studies and clinical trials.

1.7 Aims

Glioblastoma is a highly malignant tumour with a poor prognosis despite aggressive treatment. Therefore the need for new, sensitive diagnostic and prognostic biomarkers is essential for early detection and selection of the most appropriate treatment regime to improve patient outlook. The global aim of this study is to identify diagnostic and prognostic miRNA biomarkers in the serum of glioblastoma patients. Current research investigating miRNA biomarkers for glioblastoma patients often does not account for age and gender variables in miRNA expression therefore this study aimed to identify miRNA biomarkers which may be gender or age specific to provide more accurate biomarkers.

Following a review of the literature it was found that biofluids were the most attractive sample for non-invasive analysis of circulatory biomarkers for early diagnosis of GBM and prediction of prognosis. Gaps in current miRNA research include whether GBM tissue miRNA biomarkers translate into circulatory biomarkers and conversely, whether uptake of circulating exosomes containing miRNA can alter intracellular glioma and miRNA expression and subsequent extracellular release.

The first aim was to identify the effect of serum on intracellular miRNA expression and miRNA released by glioma or glial cells. This was achieved by developing an *in vitro* cell culture model to investigate miRNA expression utilising human serum, U87MG (glioma) and SVGp12 (non-cancerous, glial) cell lines to 1) define miRNA expression in the respective cell lines and 2) to determine the influence of serum type (Foetal bovine serum and human serum (from glioma patients or controls)) on cell behaviour and intracellular glioma/glial miRNA expression and miRNA released by cells into the media.

The second aim was to identify miRNAs with altered expression in the serum of glioblastoma patients and investigate the use of these miRNA as biomarkers. Initial studies determined the miRNA expression profile of glioblastoma patients grouped by gender and age: 20-39, 40-59 and 60+. Dysregulated miRNAs identified from profiling were validated in larger patient cohorts and survival data was utilised to investigate identified miRNAs as prognostic biomarkers. To address some of the pre-analytical challenges outlined in Section 1.6 serum samples utilised in this thesis were obtained pre-operatively before treatment was begun and samples were initially centrifuged prior to homogenisation in the total RNA extraction protocol to ensure the removal of contaminants such as lipids and polysaccharides.

The final aim was to identify whether dysregulated miRNAs in the serum originated from the glioblastoma tissue or from an alternative source such as leukocytes as part of an immune response. miRNA profiles of glioblastoma patient tissue and miRNA tissue data from TCGA were compared to serum miRNA expression data obtained in Chapter 4 to determine any differences in expression which might suggest an alternative source of the serum miRNAs than the glioblastoma. Further investigation of miRNA origin was performed in Chapter 5 using *In situ* hybridisation (ISH) with tissue sections selected from patients whose serum was used in Chapter 4. ISH data was compared with matched serum and tissue miRNA expression data to determine whether miRNA expression was significantly different across these three sample types.

2. Materials and Methods

2.1 Patient Samples

Human tissue, whole blood, serum and cerebrospinal fluid samples were obtained from the Brain Tumour North West tissue bank, Royal Preston Hospital. NHS ethical approval was obtained for the collection and storage of patient samples by Brain Tumour North West (REC Ref: 09/H0304/88). BTNW Tissue Bank approval and university ethical approval was obtained for the use of the patient samples in this study (Appendix 1). All patients provided written informed consent for the storage and use of their biological samples for research.

2.2 Culture of Immortalised Cell Lines

U87MG (human grade IV Glioblastoma) and SVGp12 (human non-cancerous foetal astrocytes) were obtained from the European Collection of Cell Cultures (ECACC: HPA, Salisbury, UK) and maintained in T75 flasks (Corning, Flintshire, UK) with 10 ml Eagle's minimal essential medium (EMEM, Lonza, Slough, UK), supplemented with 2mM L-Glutamine (Sigma-Aldrich, Dorset, UK) and 10 % foetal bovine serum (FBS) (Biosera, East Sussex, UK) for both cell lines. Cells were incubated at 37 ºC in 5 % CO_2 and passaged at 70 % confluency.

To passage the cells at a ratio of 1:2, medium was aspirated and cells washed with 0.1 M phosphate buffered saline pH 7.4 (PBS, Life Technologies, Paisley, UK), the PBS was then aspirated and 1 X trypsin-EDTA (Sigma-Aldrich, Dorset, UK) added and incubated with the cells for 5 minutes. After incubation, an equivalent volume of medium was added to the detached cells and transferred to new flasks containing EMEM.

2.2.1 Cell Count and Trypan Blue Assay

Trypan blue is a dye which is able to permeate necrotic and apoptotic cells while being excluded by live cells {Strober, 2001 #653}. This improves the accuracy of cell counting as it ensures only viable cells are included. Briefly, cell suspensions were diluted 1:10 with trypan blue, and the cell suspension was then placed onto the haemocytometer and the count performed. Cells containing the blue dye were not counted. The cells within five 4x4 squares were counted excluding any cells which lay across the outer

lines of the 4x4 square. An average of the cell counts for each 4x4 square was calculated then multiplied by 10 to determine the number of cells x 10^4/mL.

2.2.2 Standard Growth Curves

Growth curves for each cell line used were performed in 24 well plates to ensure that all subsequent experiments were performed in the logarithmic phase of growth. Cells were seeded at a density of 2 x 10^3/mL per well and incubated at 37 °C in 5 % CO_2. Cells were trypsinised and counted using the trypan blue exclusion method {Strober, 2001 #653} in triplicate each day over a seven day period. The growth curve was plotted and the lag and exponential phase were determined for each cell line, to determine the optimal seeding density for the serum replacement cultures.

2.2.3 Weaning of Cell Lines onto Replacement Serum

Serum from patients was obtained from the Brain Tumour North West (BTNW) tissue bank, Royal Preston Hospital. Local (Ref STEM041) and NHS ethical approval via BTNW tissue bank approval (Ref 1206) and informed patient consent was obtained for the use of patient serum samples. Cells were weaned gradually to allow acclimatisation to a new serum type and to minimise stress (van der Valk et al., 2010). Cells were cultured in T25 flasks initially with a concentration of 2 % human serum and 8 % FBS. After 48 hours of incubation the concentration of human serum was increased in increments of 5 %, 7 % and 10 %. At the same time the concentration of FBS was decreased to 5 %, 3 % and 0 %, thus the total concentration of serum in the medium was maintained at 10 %. For the serum free medium condition, cells were cultured in 25 % serum free medium and 75 % standard culture medium. The concentration of serum free medium was increased to 50 %, 75 % and 100 % at 48 hour intervals.

2.2.4 Serum Replacement Method I – Investigation of Morphological Changes

To determine the response of cell lines to serum replacement in terms of changes in morpohology, cells were cultured in T25 flasks and weaned off 10 % FBS and onto one of three conditions; 1) 10 % human serum from a pool of glioma patients (BTNW, Royal Preston Hospital), 2) a pool of 10 % non-cancerous human serum (BTNW, Royal Preston Hospital) or 3) 100 % serum free medium, a low protein, serum and animal product free substitute (Sigma-Aldrich, Dorset, UK). Samples from three patients of

each sex were obtained and pooled (Table 2.1). Weaning was performed in triplicate and three biological repeats were carried out.

Table 2.1 Serum pools of patients used in each biological repeat

Pool	Age	Sex	Diagnosis
1	60+	Male	Glioblastoma
2	60+	Female	Glioblastoma
3	60+	Male	Non-Cancerous
4	60+	Female	Non-Cancerous

2.2.5 Serum Replacement Method II – Investigation of Changes in Phenotype

To determine phenotypic changes in the cell lines following serum replacement, U87MG and SVGp12 cells were seeded at a density of 1 x 10^3 in BD Falcon 8 well chamber slides (BD Biosciences, Oxford, UK). Four wells contained medium with human serum from glioma patients and four wells contained medium with FBS as a control. For each serum weaning experiment, cells were seeded in wells in triplicate, initially with a concentration of 2 % human serum and 8 % FBS. After 48 hours incubation the concentration of human serum was increased in increments of 5 %, 7 % and 10 %. At the same time the concentration of FBS was decreased to 5 %, 3 % and 0 %.

2.2.6 Serum Replacement Method III – Investigation of MicroRNA Expression

To determine the changes in miRNA expression in response to serum replacement, cells were seeded at 2 x 10^4 cells in 96 well plates. After 24 hours incubation to allow the cells to attach, weaning was started and the medium was replaced with a concentration of 2 % human serum and 8 % FBS. The cells were subsequently weaned following the method outlined in Section 2.1.3. A 10 % FBS control was included and a medium change for the FBS control performed at the same time as the glioma and non-cancerous serum concentration was increased to ensure all cells received fresh medium. Weaning was performed in triplicate and three biological repeats were carried out.

2.2.7 Serum Replacement Growth Curves

To determine rate of growth during the process of serum replacement, U87MG and SVGp12 cell lines were seeded in 96 well plates at a density of 2 x 10^3 cells and incubated overnight. Wells were trypsinised and counted using a haemocytometer to determine the cell count for each day over a nine day period. At the same time, cells were weaned gradually onto 10 % non-cancerous human serum or 10 % serum from glioma patients following method III, Section 2.1.6. Cell counts were performed in triplicate and a trypan blue assay performed during each cell count. A 10 % FBS control was included and fresh medium was added at the time that the replacement serum concentration was increased in the other conditions. Growth curves were performed in triplicate and three biological repeats were performed.

2.3 Immunostaining

After 72 hours incubation of cells in BD Falcon 8 well chamber slides (BD Falcon, Oxford, UK) with 10 % human serum or 10 % FBS, cells were fixed and stained for neuronal and mesenchymal markers (Table 2.2).

Medium was aspirated and cells were washed three times with 0.1 M PBS. Ice cold methanol and ice cold acetone at a ratio of 1:1 was added to the chambers and fixed at -20 ˚C for 10 minutes. Following aspiration of the methanol and acetone, cells were left to air dry and subsequently rehydrated with 0.1 M PBS.

The PBS was then removed and 100 µl of primary anti-mouse antibody (Table 2.2) was added to each chamber and incubated with the lid on the slide to prevent the cells from dehydrating for 30 minutes at room temperature. The antibody was then removed and the cells washed three times with 0.1 M PBS.

A volume of 100 µl of biotinylated secondary antibody diluted 1:200 (Vectastain ABC Kit, Vector Labs, Peterborough,UK) was added to the chambers and incubated for 30 minutes with the lid on the slide. The antibody was then removed and the cells washed three times with 0.1 M PBS.

A volume of 100 µl of streptABC complex (Vectastain ABC Kit, Vector Labs, Peterborough, UK) was added to each chamber and incubated for 30 minutes at room

temperature and then removed and washed three times with 0.1 M PBS. 3,3'-diaminobenzidine (DAB) solution (Vectastain ABC Kit, Vector Labs, Peterborough, UK) was then added to each chamber and incubated for five minutes, aspirated and washed twice with distilled water. The cells were then counterstained with a few drops of hematoxylin (Sigma-Aldrich, Dorset, UK) for five minutes and rinsed with warm running water until the water became clear.

The cells were then dehydrated using 95 % ethanol followed by 100 % ethanol and finally xylene. The chamber was removed and the cells mounted with Eukitt mounting medium and a coverslip then viewed using a Nikon light microscope (Nikon, Surrey, UK) at x 10 objective.

Table 2.2 Antibodies and dilutions used for immunostaining

Marker Group	Antibody	Species	Ig Type	Marker Type	Starting Concentration	Dilution
Glial	GFAP (Dako, Cambridgeshire, UK)	Mouse	IgG1	Glial Marker	0.042 g/L	1:50
	S100B (Leica Biosystems, Milton Keynes, UK)	Mouse	IgG1	Astrocyte Marker	8 g/L	1:100
Neuronal	Nestin (Merck Millipore, Hertfordshire, UK)	Mouse	IgG1	Type IV intermediate filament protein	1 g/L	1:100
	Neurofilament (Leica Biosystems, Milton Keynes, UK)	Mouse	IgG1	Neuron Specific Cytoplasmic Filament	0.022 g/L	1:25
	Synaptophysin (Leica Biosystems, Milton Keynes, UK)	Mouse	IgG1	Presynaptic Vesicle membrane glycoprotein	0.072 g/L	1:50
	NeuN (Merck Millipore, Hertfordshire, UK)	Mouse	IgG1	Neuronal Nuclei	1 g/L	1:250
	Vimentin (Leica Biosystems,	Mouse	IgG1	Type III Intermediate filament	0.016 g/L	1:100

Mesenchymal	Milton Keynes, UK)			protein expressed in mesenchymal cells		
	Desmin (Leica Biosystems, Milton Keynes, UK)	Mouse	IgG1	Type III Intermediate filament protein expressed in mesenchymal cells	0.027 g/L	1:25
	α-SMA (Leica Biosystems, Milton Keynes, UK)	Mouse	IgG1	Myofibroblast marker expressed in mesenchymal cells	0.0045 g/L	1:25

2.4 MicroRNA Isolation

2.4.1 MicroRNA Isolation from Cell Lines

After the cell lines were weaned onto 10 % human serum (either non-cancerous or from glioma patients) as per Section 2.1.6, for 72 hours, the total isolation of RNA from the cells was performed using the *mir*Vana miRNA Isolation Kit (Life Technologies, Paisley, UK).

To maintain an RNase free environment, RNase free consumables including pipette tips and eppendorfs were utilised and workspaces were wiped down with RNase Zap (Life Technologies, Paisley, UK).

Medium was aspirated from the cells and the cells washed with 0.1 M PBS, trypsinised and counted to ensure there were 10^2 – 10^7 cells as required. The cells were transferred to a 1.5 mL RNase free centrifuge tube (Life Technologies, Paisley, UK) and centrifuged at 4500 x g for five minutes, the supernatant discarded and the cells washed in 0.1 M PBS. The cells were centrifuged again at 4500 x g for five minutes and placed on ice. The PBS was aspirated and a volume of lysis buffer was added according

to the number of cells counted, a larger volume of lysis buffer was added for a larger cell number. Cells were vortexed for 30 seconds to obtain a homogenous lysate.

Homogenate additive was added to the lysate at one tenth of the volume and vortexed for 30 seconds to mix. The cells were then incubated on ice for 10 minutes. Acid-phenol chloroform was added at a volume equal to the lysate volume before the homogenate additive was added and vortexed for 30 seconds to mix. The lysate was then centrifuged using an Eppendorf 5415D microcentrifuge (Eppendorf, Stevenage, UK) for five minutes at 16,100 x g to separate the phases and make the interphase compact. The aqueous upper phase was removed and transferred to a new centrifuge tube and the volume of liquid removed was recorded.

The elution solution was preheated to 95 ˚C over two hours, using a Grant UBD heat block (Grant Instruments, Cambridge, UK). Ethanol was added at 1.25 times the volume of the aqueous phase. A maximum volume of 700 µl of aqueous phase was pipetted onto the filter of a centrifuge collection tube. The collection tubes were centrifuged for 15 seconds at 9300 x g. The flow through was discarded from the tube and repeated with the remaining aqueous phase. Wash solution 1 was added to the collection filter and centrifuged for five seconds at 9300 x g. The flow through was then discarded and the filter replaced. Wash solution 2/3 was added and centrifuged for five seconds at 9300 x g, the flow through was discarded and the wash was repeated with solution 2/3. The flow through was discarded and the collection tubes were centrifuged for one minute at 9300 x g to remove residual fluid. The filter was transferred to a new collection tube and 100 µl of elution was added to the filter and spun for 30 seconds at 16,100 x g to recover the RNA. The concentration of the RNA was then quantified using the RNA quantification protocol in Section 2.5.

2.4.2 Exosome Isolation from Spent Media

After 72 hours incubation in the new serum conditions outlined in Section 2.1.6, exosomes were isolated using Total Exosome Isolation (from cell culture media) Reagent (Life Technologies, Paisley, UK). An equal volume of isolation reagent was added to spent medium, vortexed for 30 seconds to mix and incubated overnight at 2 ˚C. Following incubation the medium was centrifuged at 10,000 x g for one hour at 4 ˚C in an Eppendorf 5415R microcentrifuge (Eppendorf, Stevenage, UK). The medium was

then aspirated and the pellet resuspended in 0.1 M PBS. Total RNA was isolated from the pellet using the cell line isolation protocol (Section 2.3.1) and expression measured using the qRT-PCR protocol in Section 2.6 and 2.7.

2.4.3 Total RNA Isolation from Serum and Cerebrospinal Fluid

Cerebrospinal fluid samples were obtained from glioblastoma and hydrocephalus patients at the University of Athens, Greece. Local and NHS ethical approval and informed patient consent was obtained for the use of patient CSF samples. To isolate total RNA from serum and cerebrospinal fluid samples, three parts Trizol LS reagent (Life Technologies, Paisley, UK) was added to one part serum, the sample was homogenised by pipetting up and down several times and incubated at room temperature for five minutes. The samples were then centrifuged for 10 minutes at 4 ˚C at 12,000 x g, to remove extracellular matrix, polysaccharides and high molecular weight DNA.

The supernatant containing RNA was transferred to a new tube and incubated at room temperature for five minutes. A volume of chloroform equal to one quarter of the supernatant volume was added to the sample and shook vigorously by hand for 15 seconds, then incubated for 10 minutes at room temperature. The sample was then centrifuged at 12,000 x g at a temperature of 4 ˚C for 15 minutes to separate the phenol and aqueous phase.

The aqueous phase was transferred to a fresh tube and 100 % ice cold isopropanol was added and incubated for 10 minutes at room temperature. The sample was then centrifuged at 12,000 x g at a temperature of 4 ˚C for 10 minutes. The supernatant was removed and 75 % ethanol was added to the pellet and vortexed for 30 seconds to mix. The sample was then centrifuged at 7500 x g, 4 ˚C for five minutes. The supernatant was removed and air dried for two minutes and resuspended in RNase free water (Invitrogen, Paisley, UK). The RNA concentration was measured using a nanodrop as outlined in Section 2.5.

2.4.4 MicroRNA Isolation from Frozen Tissue

Local and NHS ethical approval and informed patient consent was obtained for the use of patient tissue samples. To isolate total RNA from frozen tissue samples, samples

were thawed on ice. The tissue was transferred to a 1.5 mL RNase free eppendorf tube (Life Technologies, Paisley, UK) containing Trizol LS reagent (Life Technologies, Paisley, UK) and homogenised using an OMNI tissue homogenizer (VWR, Leighton Buzzard, UK) to generate a tissue lysate. Following homogenization total RNA was extracted as outlined in Section 2.3.3 and the RNA concentration was measured using a nanodrop as outlined in Section 2.5.

2.5 Isolation of Mononuclear Cells

To determine the expression of miRNA in mononuclear cells, fresh blood samples were obtained from the BTNW tissue bank (Royal Preston Hospital, Preston, UK) and processed within two hours following collection. Local and NHS ethical approval and informed patient consent was obtained for the use of patient blood samples. Blood samples were diluted 1:1 in 0.1 M PBS and layered on top of a volume of lymphoprep (Alere LtD, Stockport, UK) double that of the blood and PBS. Samples were centrifuged for 30 minutes, 800 x g at 21 °C in an ALC PK 120R centrifuge (DJB labcare, Buckinghamshire, UK). The top layer of media was aspirated and the buffy coat layer containing mononuclear cells was removed and retained using a glass Pasteur pipette. Total RNA was extracted from the buffy coat using Trizol LS (Section 2.3.3), quantified following Section 2.5 and miRNA expression analysis was performed as outlined in Section 2.6 and 2.7.

2.6 RNA Quantification

Following extraction, total RNA concentration was measured using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Loughborough, UK) at a wavelength of 280 nm. Purity of the total RNA was determined using the 260/280 ratio, RNA samples with a 260/280 ratio of ~2.0 were considered 'pure' and used for downstream analysis (Majumdar et al., 2015).

To determine the accuracy of RNA Quantification known concentrations of a synthetic miRNA were quantified. The miRNA standard (Integrated DNA Technologies, Glasgow, UK) was diluted to produce a solution of known concentration by adding RNase free diethylpyrocarbonate (DEPC) treated water (Invitrogen, Paisley, UK) producing a stock concentration of 1 µg/µl. Subsequent serial dilutions were performed to produce a

range of concentrations; 100 ng/µl, 10 ng/µl, 5 ng/µl, 1 ng/µl and 0.1 ng/µl. The solutions were then used to determine the accuracy of the nanodrop for determining the concentration of miRNA within a solution. Measurements were performed in triplicate and the nanodrop was blanked using RNase free DEPC water (Invitrogen, Paisley, UK). Data shown in Appendix 2, total RNA concentrations above 1 ng/µl were found to be accurately measure by the nanodrop.

2.7 MiScript MiRNA PCR Array

Miscript miRNA PCR Array Brain Cancer panels (Qiagen, Manchester, UK), were used to select miRNA for a preliminary panel. Following isolation from serum samples, total RNA was reverse transcribed using the miScript RT II kit (Qiagen, Manchester, UK) and the expression level of 84 miRNAs associated with brain cancer were determined using qPCR. The *C.elegans* miR-39 miScript Primer Assay (Qiagen, Manchester, UK) was used as a control to normalise qPCR data.

Total RNA was isolated from serum and cerebrospinal fluid as outlined in Section 2.3.3 and from tissue as outlined in Section 2.3.4. The *C.elegans* miR-39 miScript Primer Assay) was diluted to a final concentration of 1.6 $x10^8$ copies/µl (Table 2.3) and added to the sample following homogenisation and lysis. Total RNA was diluted to a final concentration of 12.5 ng/µl and then reverse transcribed. The master mix components excluding the reverse transcriptase mix were thawed at room temperature and the master mix was prepared following Table 2.4. The reverse transcriptase was removed from -20 ˚C immediately before adding to the master mix and then immediately replaced into -20 ˚C to maintain integrity of the enzyme. The master mix was mixed by pipetting, centrifuged at 2000 x g for five seconds in a Technico mini centrifuge (Fisher Scientific, Loughborough, UK) stored on ice and then incubated at 37 ˚C for 60 minutes followed by 95 ˚C for five minutes.

Table 2.3: Dilution and Final Concentration of C. *elegans* miR-39 miScript Primer Assay.

	Dilution	Concentration (Copies/µl)
Initial Stock	300 µl DEPC treated H_2O + 10 pmol lyophilised sample	2 x 10^{10}
Dilution	4 µl stock + 16 µl RNase-free water	4 x 10^9
Working Solution	2 µl of dilution + 48 µl RNase	1.6 $x10^8$

free water

Table 2.4: Reverse Transcription Master Mix, Components and Final Working Concentrations.

Component	Volume	Final Concentration
5 X miScript HiSpec Buffer	4 µl	1 X
10 X miScript Nucleics Mix	2 µl	1 X
RNase-free water	Variable	-
MiScript Reverse Transcriptase Mix	2 µl	-
Template RNA	Variable	12.5 ng/µl
Total Volume	20 µl	-

Following reverse transcription cDNA was diluted 1:10 in RNase free DEPC water (Life Technologies, Paisley, UK) prior to qPCR. The qPCR master mix components were thawed at room temperature and pipetted following Table 2.5. The brain cancer panels were thawed at room temperature, and 25 µl of reaction mix was added to each well and the plate sealed with optical adhesive film. The plate was then centrifuged at 1000 x g for one minute to ensure all the master mix was at the bottom of the well. The *C.elegans*, reverse transcription control and positive PCR control wells within the brain cancer panels were utilised in this study.

The qPCR reaction was performed on an Applied Biosystems 7500 Real Time PCR System in standard mode using the parameters outlined in Table 2.6 and a dissociation analysis step added at the end of the run.

Table 2.5: qPCR Master Mix and Final Working Concentration.

Component	Volume (µl)	Final Concentration
2 X QuantiTect SYBR Green PCR Master Mix	1375	1 X
10 X miScript Universal Primer	275	1 X
RNase free water	1000	-
Template cDNA (diluted)	100	-
Total Volume	2750	-

Table 2.6: qPCR Parameters.

	Enzyme Activation	**Hold 15 minutes**	**95 ˚C**
PCR - 40 Cycles	**Denature**	Hold 15 seconds	94 ˚C
	Anneal	Hold 30 seconds	55 ˚C
	Extend	Hold 34 seconds	70 ˚C
Melt curve analysis – 1 Cycle	**Dissociation**		60-95 ˚C

The data were analysed using the miScript miRNA PCR Array Data Analysis tool (SABiosciences) available online at http://pcrdataanalysis.sabiosciences.com/mirna. Threshold cycle (Ct) values were normalised using the *C.elegans* miR-39 miScript Primer Assay (Qiagen, Manchester, UK). Normalisation was performed by subtracting the Ct value of the *C.elegans* spike in from the target miRNA Ct value. Each miRNA was scored either A, B or C by the miScript data analysis tool based on the average Ct to determine whether the calculated fold change was representative of the actual fold change within the sample (Table 2.7). 'A' being representative of the actual fold change and 'B' suggesting a variation in fold change between samples which may not be representative of the actual fold change. MiRNAs were scored 'C' if the average Ct of either the target sample or the control sample was beyond the defined cut off of 35 cycles, making the calculated fold change invalid. Following analysis using the miScript miRNA PCR Array Data Analysis tool, data was examined and miRNAs with a fold change scored as a 'C' were omitted from the study.

Table 2.7 Fold Change Scoring system defined by the miScript data analysis tool.

Score	**Definition**
A	This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30).These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result.
B	This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples.

C	This gene's average threshold cycle is either not determined or greater than the defined cut-off (default 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.

2.8 NCode miRNA First-Strand cDNA Synthesis

For subsequent analysis of miRNA expression following the use of the miScript arrays, total RNA was polyadenylated and reverse transcribed using the NCode miRNA First-Strand cDNA synthesis kit (Life Technologies, Paisley, UK). Following extraction of total RNA as outlined in Section 2.3.3, the samples were polyadenylated and reverse transcribed using the NCode miRNA First-Strand cDNA Synthesis kit. RNA samples were diluted in RNase free DEPC water to 200 ng. Stock 10 mM dATP was diluted 2:50 with 1 mM Tris, pH 8.0. A master mix for Polyadenylation was prepared as detailed in Table 2.8.

Table 2.8: Polyadenylation Reaction Master Mix

Component	Volume (µl)	Final Concentration
5 X miRNA Reaction Buffer	5	1 X
25 mM $MnCl_2$	2.5	2.5 mM
Diluted ATP	1	0.8 mM
Poly A Polymerase	0.5	-
Diluted total RNA	16	12.5 ng/µl
Total	**25**	-

The samples were incubated in a Grant UBD heat block (Grant Instruments, Cambridge, UK), pre-heated to 37 °C, for 15 minutes. Immediately following incubation, the samples were reverse transcribed. A volume of 4 µl of polyadenylated RNA was used in the following cDNA synthesis reaction. The RNA samples were combined with annealing buffer and universal RT primer in the volumes outlined in Table 2.9.

Table 2.9: Initial cDNA Synthesis Master Mix

Component	Volume (µl)	Final Concentration

Polyadenylated RNA	4	12.5 ng/µl
Annealing Buffer	1	-
Universal RT Primer (25 µM)	3	9.375 µM
Total	**8**	-

The tubes were incubated for five minutes in a Veriti Thermal cycler (Life Technologies, Paisley, UK) pre-heated to 65 °C and then placed on ice for one minute. Further components were added to the tubes, shown in Table 2.10 and incubated at 50 °C for 50 minutes followed by five minutes at 85 °C to stop the reaction. The cDNA was stored at -20 °C before proceeding to qPCR.

Table 2.10: Secondary cDNA Master Mix

Component	Volume (µl)	Final Concentration
2 X First-strand reaction mix	10	1 X
SuperScript III RT enzyme mix	2	-
Total	**12**	-

2.9 GoTaq SYBR Green qPCR Reaction

Following first strand cDNA synthesis as outlined in Section 2.7, cDNA samples were diluted 1:10 in RNase free DEPC water. The qPCR master mix was prepared as outlined in Table 2.11 using the universal reverse primer provided with the NCode miRNA First-Strand cDNA synthesis kit and miRNA specific forward primers outlined in Section 2.9.1 (Integrated DNA Technologies, Glasgow, UK).

Table 2.11: GoTaq SYBR Green qPCR Master Mix

Component	Volume (µl)	Final Concentration

2 X qPCR master mix	12.5	1 X
Forward primer, 10 µM	0.5	200 nM
Universal reverse primer, 10 µM	0.5	200 nM
RNase free water	6.5	-
Total	**20**	-

The cDNA was diluted 1:5 with qPCR master mix and added to each well of a MicroAmp Optical 96-Well Reaction Plate (Life Technologies, Paisley, UK) the plate was sealed with MicroAmp Optical Adhesive Film (Life Technologies, Paisley, UK) and centrifuged for one minute at 1000 x g.

The qPCR reaction was performed on an Applied Biosystems 7500 Real Time PCR System (Life Technologies, Paisley, UK) in standard mode using the parameters outlined in Table 2.12 and a dissociation analysis step added at the end of the run. Data was analysed using the ΔCt method and normalised using the *C.elegans* miR-39 miScript Primer Assay (Qiagen, Manchester, UK).

Table 2.12: qPCR run parameters

	Enzyme Activation	**Hold 2 minutes**	**95 ˚C**
PCR - 40 Cycles	**Denature**	Hold 15 seconds	95 ˚C
	Anneal/Extend	Hold 60 seconds	60 ˚C
Melt curve analysis – 1 Cycle	**Dissociation**		60-95 ˚C

2.9.1 Primer Design

Specific forward primers (Integrated DNA Technologies, Glasgow, UK), were designed using sequences obtained from miRBase available online www.miRBase.org (Table 2.13).

2.10 MiRNA Expression in TCGA Dataset

To determine the tissue expression of miRNA in a larger patient cohort of 558 patients, microarray data was analysed from the TCGA by Josie Hayes, Leeds Institute of Molecular Medicine. Log fold change expression in glioblastoma patients compared to patients without glioblastoma was determined and a comparison of expression and age was determined using Pearson's correlation. Survival analysis was performed using both cox regression and log-rank above and below the median.

Table 2.13 Specific forward primers designed and utilised in this study.

Primer	Sequence
hsa-let-7-5p	5'-TGA GGT AGT AGG TTG TAT AGT T-3'
hsa-miR-7-5p	5'-TGG AAG ACT AGT GAT TTT GTT GT-3'
hsa-miR-9-3p	5'-ATA AAG CTA GAT AAC CGA AAG T-3'
hsa-miR-15b-5p	5'-TAG CAG CAC ATC ATG GTT TAC A-3'
hsa-miR-16-5p	5'-TAG CAG CAC GTA AAT ATT GGC G-3'
hsa-miR-17-5p	5'-CAA AGT GCT TAC AGT GCA GGT AG-3'
hsa-miR-18a-5p	5'-TAA GGT GCA TCT AGT GCA GAT AG-3'
hsa-miR-19a-3p	5'-TGT GCA AAT CTA TGC AAA ACT GA-3'
hsa-miR-19b-3p	5'-TGT GCA AAT CCA TGC AAA ACT GA-3'
hsa-miR-20a-5p	5'-TAA AGT GCT TAT AGT GCA GGT AG-3'
hsa-miR-21-5p	5'-TAG CTT ATC AGA CTG ATG TTG A-3'
hsa-miR-23a-3p	5'-ATC ACA TTG CCA GGG ATT TCC-3'
hsa-miR-25-3p	5'-CAT TGC ACT TGT CTC GGT CTG A-3'
hsa-miR-26a-5p	5'-TTC AAG TAA TCC AGG ATA GGC T-3'
hsa-miR-29b-3p	5'-TAG CAC CAT TTG AAA TCA GTG TT-3'
hsa-miR-29c-3p	5'-TAG CAC CAT TTG AAA TCG GTT A-3'
hsa-miR-30b-5p	5'-TGT AAA CAT CCT ACA CTC AGC T-3'
hsa-miR-30c-5p	5'-TGT AAA CAT CCT ACA CTC TCA GC-3'
hsa-miR-34a-5p	5'-TGG CAG TGT CTT AGC TGG TTG T-3'
hsa-miR-92a-3p	5'-TAT TGC ACT TGT CCC GGC CTG T-3'
hsa-miR-93-5p	5'-CAA AGT GCT GTT CGT GCA GGT AG-3'

hsa-miR-101-3p	5’-TAC AGT ACT GTG ATA ACT GAA-3’
hsa-miR-148a-3p	5’-TCA GTG CAC TAC AGA ACT TTG T-3’
hsa-miR-150-5p	5’-TCT CCC AAC CCT TGT ACC AGT G-3’
hsa-miR-181a-5p	5’-AAC ATT CAA CGC TGT CGG TGA GT-3’
hsa-miR-181b-5p	5’-AAC ATT CAT TGC TGT CGG TGG GT-3’
hsa-miR-185-5p	5’-TGG AGA GAA AGG CAG TTC CTG A-3’
hsa-miR-191-5p	5’-CAA CGG AAU CCC AAA AGC AGC UG-3’
hsa-miR-320a	5’-AAA AGC TGG GTT GAG AGG GCG A-3’
hsa-miR-328-3p	5’-CTG GCC CTC TCT GCC CTT CCG T-3’
hsa-miR-451a	5’-AAA CCG TTA CCA TTA CTG AGT T-3’
hsa-miR-486-5p	5’-TCC TGT ACT GAG CTG CCC CGA G-3’
cel-miR-39-3p	5’-TCA CCG GGT GTA AAT CAG CTT G-3’

2.11 *In Situ* Hybridisation

2.11.1 Preparation of Buffers and Stock solutions

To determine the localisation of microRNAs within tumour tissue sections, *in situ* hybridisation (ISH) was performed using the miRCURY LNA microRNA ISH Optimization Kit (FFPE) (Exiqon, UK) and the ISH protocol outlined by Gerard Nuovo (Nuovo, 2010). Prior to the ISH experiment proteinase-K (Exiqon, UK) was reconstituted in 10 mM Tris-HCl (pH 7.5) to produce a 20 mg/mL stock which was aliquoted and stored at - 20 °C. Proteinase-K buffer, 0.2 X saline-sodium citrate (SSC) solution (Sigma-Aldrich, Dorset, UK) and 0.1 % PBS-Tween (PBS-T, pH 7.4) were also prepared prior to the ISH experiment as outlined in Table 2.14 and autoclaved to minimise RNase activity.

Table 2.14: Buffers and solutions prepared prior to ISH

Buffer	Component	Volume (mL)	Final Concentration
Proteinase-K Buffer	RNase free water	500	-
	1 M Tris-HCl (pH 7.4)	2.5	
	5 M NaCl	0.1	
0.2 X SSC	RNase free water	495	-
	20X SSC	5	0.2 X

PBS-T pH 7.4	PBS	500	-
	Tween-20	0.5	0.1 %

Prior to the ISH experiments stocks of the hybridisation solution containing each probe were prepared. To prepare the hybridisation mix, 2 X microRNA ISH buffer (miRCURY LNA microRNA ISH Optimization Kit) was diluted 1:1 with RNase free DEPC water to produce a final concentration of 1 X. The double-DIG-labelled probes were diluted in a 2 mL non-stick RNase free tube (Life Technologies, Paisley, UK). The probes were denatured at 90 °C for 4 minutes using a Grant UBD heat block (Grant Instruments, Cambridge, UK) and 1 X microRNA ISH buffer was added to each of the tubes to dilute the probes to the appropriate final concentration. The probe stocks were aliquoted and stored at - 20 °C.

2.11.2 Optimisation of Proteinase-K Step

Optimisation of the proteinase-K concentration and duration of treatment was performed using the LNA U6 snRNA double-digoxygenin (DIG) labelled detection probe (Exiqon, UK). The LNA U6 snRNA probe was diluted in microRNA ISH buffer in a 2 mL non-stick RNase free tube (Life Technologies, Paisley, UK) to produce a range of concentrations between 0.1 and 2 nM as outlined in Table 2.15.

Table 2.15: Concentrations of LNA U6 snRNA Probe used for Proteinase-K Optimisation

Dilution Factor	Probe Volume (µl)	1 X ISH Buffer Volume (mL)	Final Concentration (nM)
1:5000	0.4	2	0.1
1:1000	2	2	0.5
1:500	4	2	1
1:333	6	2	1.5
1:250	8	2	2

Immediately before proteinase-K treatment of slides, proteinase-K stock solution was added to the proteinase-K buffer to produce a final concentration of 15 µg/mL. Diluted proteinase-K was added to each slide to cover the tissue section and incubated for 10

minutes at 37 °C in a HYBAID Micro-4 hybridisation oven (VWR, Leighton Buzzard,UK). Following negative probe signal from sections incubated with 15 µg/mL proteinase-K for 10 minutes, a second optimisation experiment was performed with 15 µg/mL proteinase-K incubated at 37 °C for 20 minutes, which produced a signal using the U6 probe.

2.11.3 Optimisation of Hybridisation Temperature and Incubation Time

Following optimisation of the proteinase-K step, optimisation of the hybridisation temperature for the U6 and miR-21 positive control probes was performed. The U6 snRNA probe was diluted to a final concentration of 10 nM. The miR-21 positive control probe and the scrambled negative control probe were both diluted to a final concentration of 40 nM (Table 2.16). The hybridisation mix was added to each slide to cover the section, a sterile cover glass was added and sealed with fixogum and hybridised overnight at 37 °C or 4 °C.

Table 2.16 Probe dilutions and final concentrations for hybridisation temperature optimisation.

Probe	Dilution Factor	Probe Volume (µl)	1 X ISH Buffer Volume (mL)	Final Concentration (nM)
U6 snRNA	1:250	4	1	10
miR-21	1:625	3.2	2	40
Scrambled	1:625	3.2	2	40

Following optimisation of the hybridisation temperature, optimisation of incubation times for anti-DIG AP and NBT-BCIP was performed. Slides were incubated for one hour with anti-DIG AP and one hour with NBT-BCIP, two hours with anti-DIG AP and one hour with NBT-BCIP and two hours with anti-DIG AP and two hours with NBT-BCIP.

2.11.4 Optimised *In Situ* Hybridisation of Target MiRNAs

Prior to ISH of miRNA biomarkers optimisation of proteinase-K concentration (Section 2.10.2), duration of proteinase-K treatment (Section 2.10.2) and optimisation of probe hybridisation temperature (Section 2.10.3) was carried out.

Local and NHS ethical approval and informed patient consent was obtained for the use of patient tissue section samples. Glioblastoma FFPE tissue sections (BTNW tissue bank, Royal Preston Hospital, UK) were deparrafinised for five minutes in histoclear (Fisher Scientific, Loughborough, UK) and five minutes in 100 % ethanol. Following deparaffinisation, slides were air dried at room temperature.

Immediately before proteinase-K treatment of slides, proteinase-K stock solution was added to proteinase-K buffer to produce the final concentration of 15 µg/ml as optimised in Section 2.10.2. Diluted proteinase-K was added to each slide to cover the section and incubated for 20 minutes at 37 °C in a HYBAID Micro-4 hybridisation oven (VWR, Leighton Buzzard, UK). Slides were washed briefly in RNase free water followed by 100 % ethanol and air dried at room temperature.

Hybridisation mix was added to each slide to cover the section and a sterile cover glass was applied and sealed with fixogum rubber cement (Amazon, London, UK). Slides were incubated at 65 °C for five minutes and subsequently incubated overnight at 37 °C.

Immediately prior to continuing the ISH experiment antibody blocking solution, antibody diluent solution and anti-DIG reagent were prepared. To prepare the antibody blocking solution sheep serum (Sigma Aldrich, Dorset, UK) was added to 0.1 % PBS-T (pH 7.4) to produce a final concentration of 2 % sheep serum. 5 mL of blocking solution was aliquoted into a new tube for the antibody diluent solution. Stock 30 % BSA was added to the blocking solution to produce a final concentration of 1 %. To prepare the antibody diluent solution, 30 % BSA was added to 0.1 M PBS to produce a final concentration of 1 % BSA. Sheep-anti-DIG-AP was diluted 1:200 in antibody dilutant solution.

Following overnight hybridisation, the cover glass was detached and slides were placed in 0.2 X SSC in a glass jar placed in a Grant JB1 water bath (Fisher Scientific, Loughborough, UK) set at 50 °C for 10 minutes.

A hydrophobic barrier was made around each section and antibody blocking solution was added to each slide and incubated for 15 minutes at room temperature. The blocking solution was removed and anti-DIG-reagent was added to each slide and incubated for two hours at 37 °C in humidifying conditions in a HYBAID Micro-4

hybridisation oven (VWR, Leighton Buzzard, UK). The slides were subsequently washed for three minutes in PBS-T prior to application of the substrate.

Immediately prior to use alkaline phosphatase (AP) substrate was prepared by dissolving one nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyl-phosphate (NBT-BCIP) tablet (Roche, Hertfordshire, UK) in 10 mL of double distilled water pre-heated to 37 °C. Levamisol stock (Sigma-Aldrich, UK) was added to the NBT-BCIP to produce a final concentration of 0.2 mM. The AP substrate was applied to each section and incubated for one hour at 37 °C in a HYBAID Micro-4 hybridisation oven (VWR, Leighton Buzzard, UK), the slides were protected from light. The slides were washed for one minute with running tap water.

Nuclear fast red (Vector Laboratories, UK) was added to each slide to cover the section for one minute for nuclear counter staining. Slides were placed in a glass jar and rinsed with running tap water for 10 minutes, dipped in 100 % ethanol and allowed to air dry.

Slides were dipped in histoclear and subsequently mounted with one drop of Eukitt quick-hardening mounting medium (Sigma-Aldrich, Dorset, UK) and a sterile glass cover slip. The precipitate was allowed to settle overnight and viewed with a Nikon Eclipse E200 Phase Contrast microscope (Nikon, Surrey, UK) on the brightfield setting at x10 and x100 objectives the following day.

2.12 Power Analysis

To ensure any miRNA biomarkers identified were statistically significant, power analysis was performed to determine the sample size required for 80 % power. Power analysis was performed using the GraphPad Statmate software (San Diego, USA). Standard deviations for each of the miRNA biomarkers were used to determine the sample number required to detect a difference between means of 1 Ct value.

2.13 Data Analysis

Data were analysed using GraphPad Prism 5.0 statistical software (San Diego, USA). Raw ΔCt values for both target and control data were used for statistical analysis. Following statistical analysis control ΔCt values were presented as a standard fold change value of one in figures with no standard deviation. Where sample numbers

permitted, a Kolmogorov Smirnov test was used to determine whether data were normally distributed. Data consisting of two groups were analysed by student's t-test with Levene's F test or the non-parametric Mann-Whitney U test. Data consisting of three groups were analysed by one-way ANOVA with Tukey *post-hoc* test or non-parametric Kruskal-Wallis with Dunn's *post-hoc* test. Data consisting of paired groups were analysed by two-way ANOVA with Tukey *post-hoc* test. Following analysis of both one-way and two-way ANOVA using the Tukey *post-hoc* test, data was tested for multiple variances by adjusting the *p*-value for the number of groups. Survival data were analysed using Log-rank (Mantel Cox) test to determine whether differences between survival curves were significantly different. The TCGA dataset was analysed by Josie Hayes (Leeds Institute of Molecular Medicine) using log-rank above and below the median. Correlations between miRNA expression and age were determined using Pearson's correlation. All data except the miSCript brain cancer array data in Chapters 3 and 5 were displayed as means of triplicate experiments + SD. A *p*-value of < 0.05 was considered significant.

3. Effect of Serum Replacement on Immortalised Cell Lines

3.1 Introduction

Foetal bovine serum (FBS) is the standard serum type used to supplement basal medium in the culture of immortalised cell lines including U87MG and SVGp12 cells. FBS contains high levels of hormones, proteins and growth factors required to stimulate growth and proliferation of cell lines (Zheng et al., 2006). Although commonly used for the culture of numerous cell lines, FBS is poorly defined and the exact composition is unknown. The composition of FBS can vary between different batches and therefore can have varying effects on the growth of cell lines (Zheng et al., 2006). Whilst not used for the maintenance of immortalised cell lines, human serum is one of a variety of serum types that is used for the *ex vivo* expansion of mesenchymal or human embryonic stem cells (hESC) prior to *in vivo* transplantation (Mannello and Tonti, 2007). Studies have shown that the culture of hESC with human serum encourages the maintenance of the hESC karyotype and pluripotency even after prolonged culture (Mannello and Tonti, 2007).

During disease, factors within human serum may change in abundance or the presence or absence of certain components may occur (Tirumalai et al., 2003). One such component is exosomes, small lipoprotein vesicles which are present in the serum of both healthy and diseased individuals. Exosomes are believed to be a method of intercellular communication (Colombo et al., 2014) and are capable of immune modulation (Blin and Fitzgerald, 2015) and manipulation of tumour microenvironments (Milane et al.). Exosomes contain mRNA, miRNA and proteins which can be released and taken up by neighbouring cells (Meckes et al., 2010). The specific contents of exosomes often reflect the cell of origin therefore those derived from tumour cells contain proteins and RNA with the ability to promote tumour characteristics in recipient cells including invasion and proliferation (Meckes et al., 2010).

Glioblastoma cells release exosomes containing angiogenic proteins as well as mRNA and miRNA which stimulate tubule formation in recipient endothelial cells (Skog et al., 2008). The release of glioblastoma specific exosomes can therefore play an important role in stimulating invasion and metastasis (Katakowski et al., 2010), a vital characteristic which contributes to the malignancy of glioblastoma and hinders successful treatment (Demuth and Berens, 2004).

The release and uptake of exosomes has been demonstrated *in vitro* in U87MG and U251 glioblastoma cell lines (Katakowski et al., 2010). Furthermore, studies have shown that recipient cells incorporate exosomal miRNA into cellular pathways resulting in altered protein expression (Katakowski et al., 2010). Short-term glioblastoma derived from primary cells are also able to influence protein expression of endothelial cells in culture and promote angiogenesis (Skog et al., 2008). In addition, isolation of exosomes from primary glioblastoma cells and incubation with U87MG cells caused an increase in proliferation compared to untreated U87MG cells (Skog et al., 2008), showing exosomes of *in vivo* origin can influence the behaviour of immortalised cell lines. The aforementioned studies investigating the function of circulating miRNAs in cell lines have utilised the standard FBS culture conditions, however as FBS is of bovine origin this may affect data collected. Cell culture using

human serum as a supplement may, therefore, provide a more representative model for miRNA function in humans and could provide more accurate data.

The aim of the current study was to establish a human serum based cell culture model which could be used to investigate miRNA expression. Once established, the behaviour of U87MG and SVGp12 cells in culture with glioblastoma patient serum and non-cancerous human serum was investigated. Further studies were performed to determine whether human serum could alter the miRNA expression of immortalised cell lines and whether the resulting phenotype differed between cells cultured in serum from cancerous and non-cancerous subjects. It was hypothesised that the composition FBS, human serum from glioma patients and non-cancerous control patients would differ and therefore alter the growth behaviour and expression of selected miRNAs in the cell lines.

3.2 Results

3.2.1 Culture of U87MG and SVGp12 cells in replacement serum resulted in increased cell number

To determine whether the weaning of cell lines off standard FBS and onto human serum had an effect on growth characteristics, growth curves were performed during the weaning phase. Cells were initially seeded with 10 % FBS and subsequently weaned onto increasing concentrations of human serum pooled from three glioblastoma patients or three control patients in increments of 2 %, 5 %, 7 % and 10 % whilst decreasing the FBS at increments of 8 %, 5 % , 3 % and 0 % to maintain the total serum concentration at 10 % (Section 2.1.7). Cells were cultured for 48 hours in each serum concentration to allow completion of one cell cycle of growth before increasing the replacement serum concentration (Section 2.1.7).

The growth of U87MG cells in all three serum conditions was not significantly different ($p > 0.05$) until 48 hours culture in 7 % replacement serum; after which there was a significant increase in cell number in both the 7 % serum from glioblastoma patients and 7 % serum from non-cancerous patients compared to cells cultured in 10 % FBS ($p < 0.05$) (Figure 3.1). There was also a significant increase in cell number in the glioma patient serum compared to the non-cancerous subjects after 48 hours incubation. (Figure 3.1D). Following culture in 10 % replacement serum, U87MG cells cultured in 10 % glioma patient serum showed the greatest increase in cell number ($p < 0.001$) compared to those cultured in FBS, followed by U87MG cells cultured in 10 % non-cancerous human serum ($p < 0.01$).

A similar trend in growth was observed with the SVGp12 cell line where cell number was not significantly different until after 48 hours incubation with 7 % replacement serum (Figure 3.2). In contrast to the U87MG cell line, SVGp12 cells grown in 7 % non-cancerous human serum and 3 % FBS showed the greatest increase in cell number compared to both the 7 % glioma patient serum and 10 % FBS conditions ($p < 0.001$) (Figure 3.2D). Following culture in 10 % replacement serum SVGp12 cells incubated in 10 % glioma patient serum showed a similar cell number to those cultured in 10 % FBS and no significant difference in cell number was observed between the glioma patient serum and FBS conditions (Figure 3.2D).

Comparison of the two cell lines cultured in the same serum condition showed that cell numbers for U87MG were significantly higher than SVGp12 following 48 hours incubation with 7 % replacement serum and in the 10 % FBS control condition. Cell numbers were not significantly different for both cell lines in the same condition prior to incubation in 7 % replacement serum.

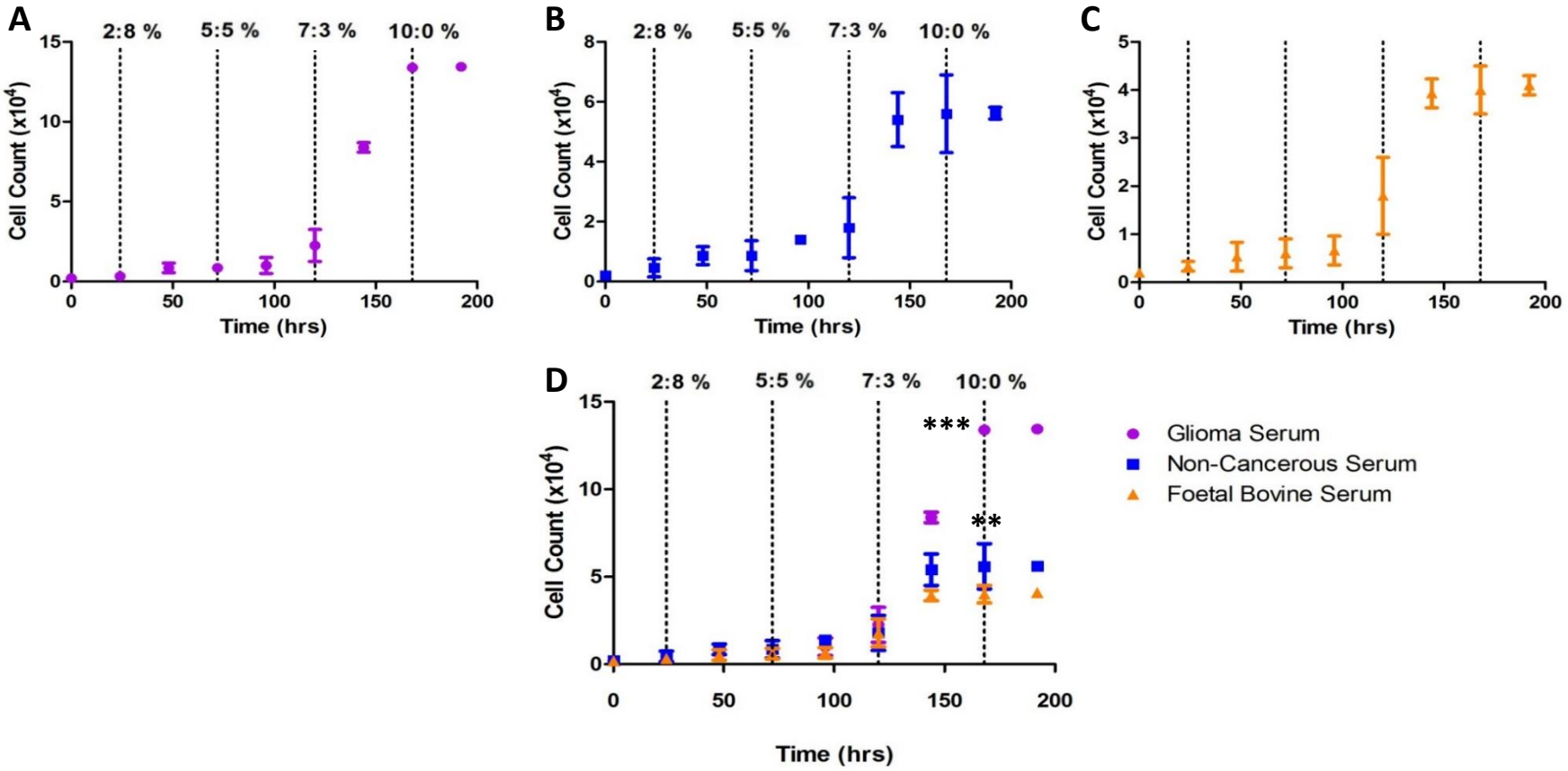


Figure 3.1 Growth of U87MG cells during serum replacement. A) U87MG cells cultured in glioblastoma patient serum. B) U87MG cells cultured in non-cancerous human serum. C) U87MG cells cultured in FBS. D) Combined growth curves of all serum types. Cell number for U87MG cells cultured in glioblastoma patient serum was signifcantly higher compared to those cultured in non-cancerous human serum and FBS, ***$p < 0.001$. U87MG cells cultured in non-cancerous human serum had significantly higher cells numbers compared to FBS, **$p < 0.01$. Data shown as mean of triplicate experiments (n=9) with SD, analysed by two way ANOVA with Tukey *post-hoc* test. Ratios are shown as percentage of replacement serum to percentage of FBS.

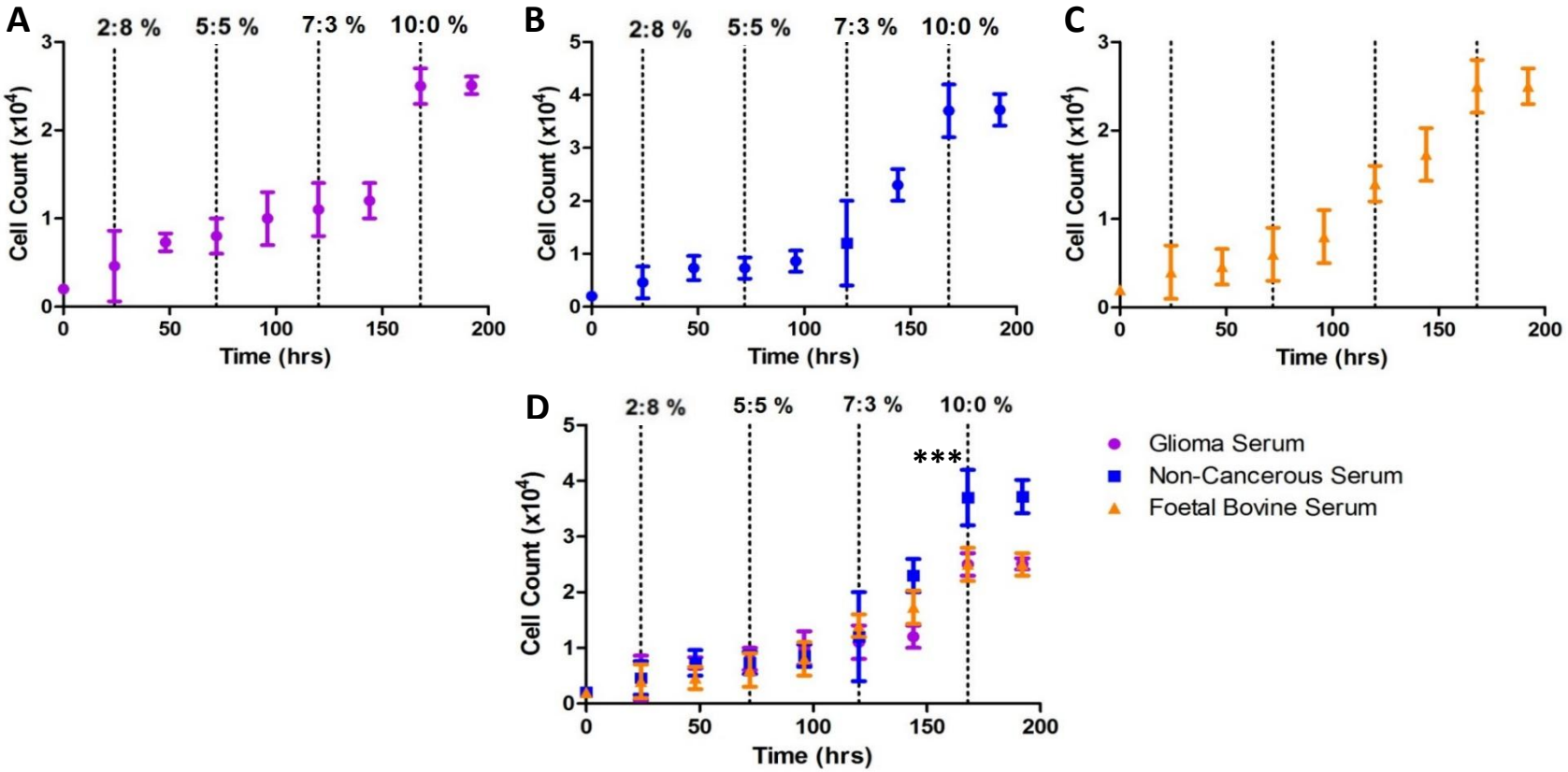


Figure 3.2 Growth of SVGp12 cells during serum replacement. A) SVGp12 cells cultured in glioblastoma patient serum. B) SVGp12 cells cultured in non-cancerous human serum. C) SVGp12 cells cultured in FBS. D) Combined growth curves of all serum types. There was no significant difference in cell number between cells cultured in glioblastoma patient serum and those cultured in FBS. SVGp12 cells cultured in non-cancerous human serum had a significantly higher cell number compared to those cultured in glioblastoma patient serum and FBS. Data shown as mean of triplicate experiments (n=9) with SD, analysed by two way ANOVA with Tukey *post-hoc* test ***$p < 0.001$. Ratios are shown as percentage of replacement serum to percentage of FBS.

3.2.2 The culture of U87MG and SVGp12 cells in replacement serum resulted in morphological changes

The process of serum replacement was observed to have a morphological effect on U87MG cells. When cultured in the presence of either non-cancerous human or glioblastoma patient serum, the cells underwent changes in appearance (Figure 3.3). Serum-free medium was used as a control in morphology studies to determine the morphological response of cells to an absence of serum.

The morphology of U87MG cells in non-cancerous human serum displayed spiky processes were star-like (Figure 3.3E-H). In human serum obtained from glioma patients, U87MG cells exhibited a more arranged morphology forming branched linear structures similar to an epithelial phenotype (Figure 3.3A-D). Conversely, the appearance of SVGp12 cells did not visibly alter when cultured in both cancerous and non-cancerous human serum compared to those cultured in 10 % FBS (Figure 3.4A-H).

Changes in morphology of the U87MG cells was observed from 48 hours culture in 3 % replacement serum and 7 % FBS (Figure 3.3A). The linear structures of an epithelial phenotype were observed most frequently after 48 hours incubation with 10 % glioma serum alone (Figure 3.3D). In addition to the observed morphological changes, the culture of U87MG cells in human serum led to an increase in spheroid formations. These spheroids were mainly attached to the flask and those in the glioma patient serum were also incorporated into the linear structures which radiated from a central spheroid (Figure 3.3B). The changes in morphology were most pronounced after 48 hours culture in 10 % glioma patient serum (Figure 3.2D).

The culture of cells in varying concentrations of serum free medium also led to a change in morphology. Non-viable cells for both U87MG and SVGp12 were observed as dark, detached, rounded cells (Figure 3.3I and 3.4I). In addition, the confluency of both cell lines was also greatly reduced compared to the other serum conditions. The culture of SVGp12 and U87MG cells in serum free medium led to the formation of both detached and attached spheroids (Figure 3.3 and 3.4).

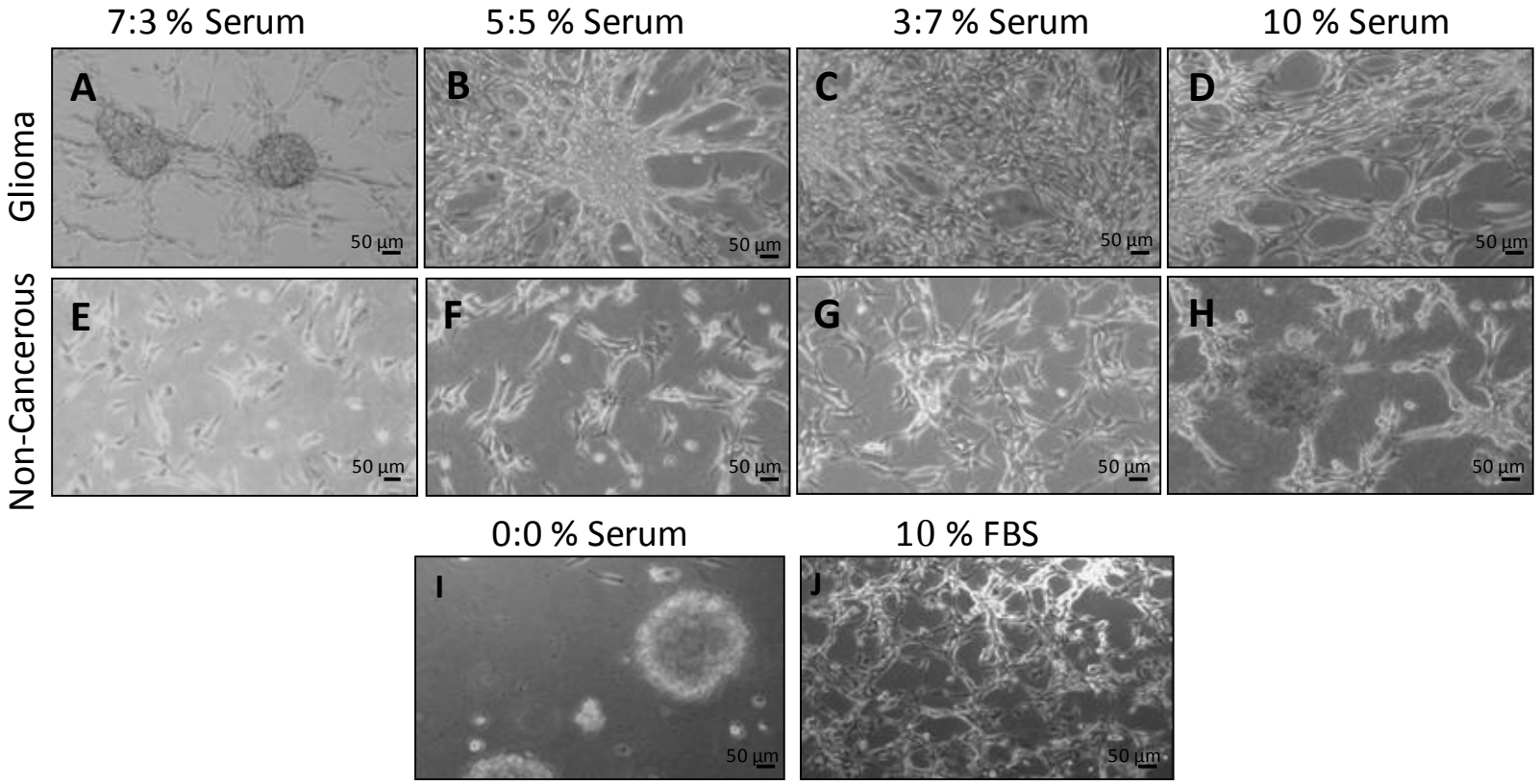


Figure 3.3 Morphology of U87MG cells cultured in glioma patient serum and non-cancerous human serum. The culture of U87MG cells in glioblastoma patient serum was shown to cause a change in morphology with the formation of attached spheroids and elongated uniform arrangements of cells (A-D), seen most prominently following culture in 10 % human serum (D). U87MG cells cultured in non-cancerous serum exhibited a 'star-like' appearance similar to that of fibroblasts (E-H). Following culture in 10 % non-cancerous serum, spheroids were also observed (H). U87MG cells cultured in serum-free medium were observed to form detached spheroids (I). (J) U87MG cells cultured in standard 10 % FBS conditions. Images taken following 48 hrs culture in replacement serum concentrations at x10 magnification.

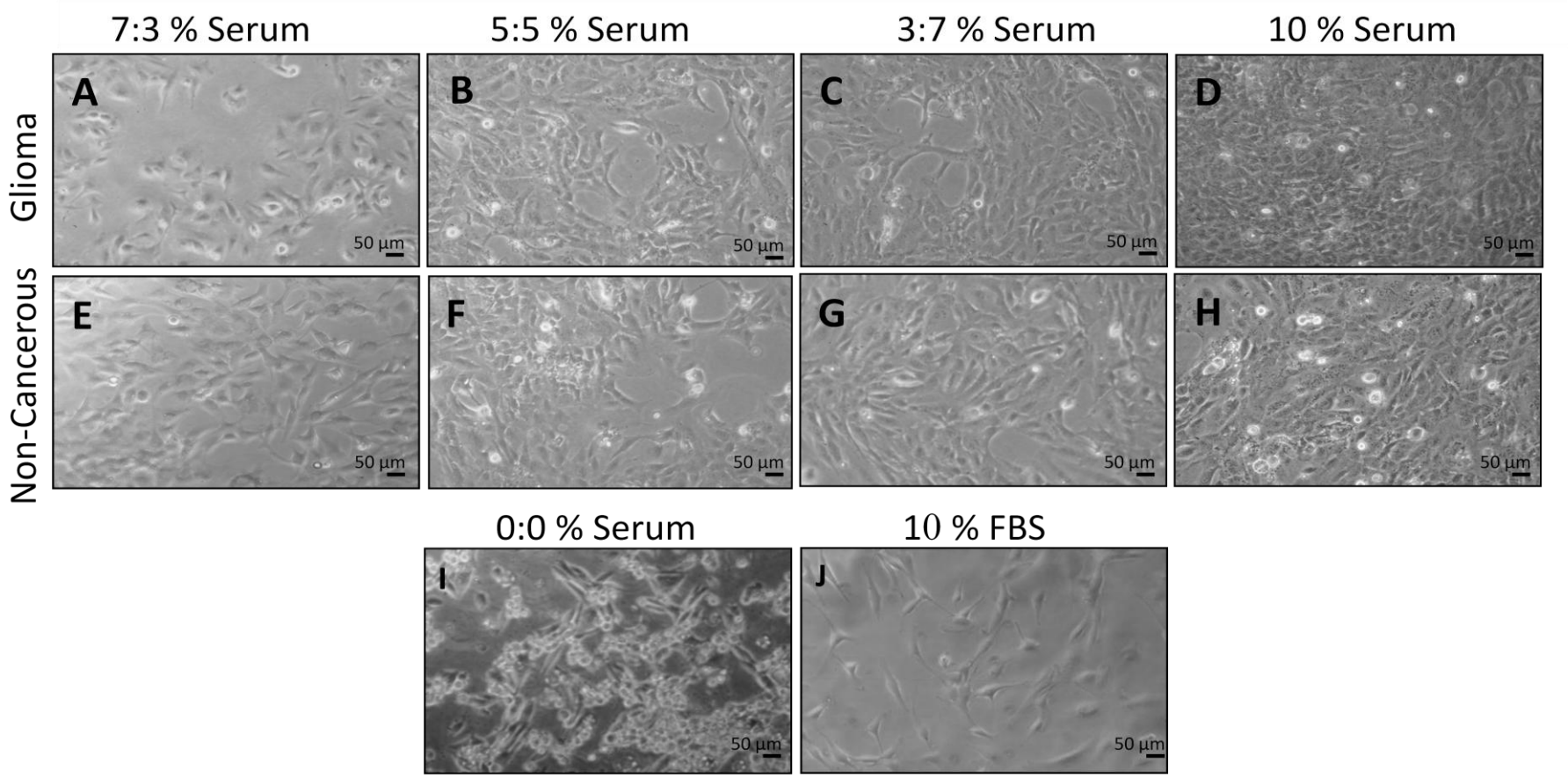


Figure 3.4 Morphology of SVGp12 cells cultured in 10 % glioma patient serum and 10 % non-cancerous human serum. Culture of SVGp12 in both glioma patient serum (A-D) and non-cancerous human serum (E-H) did not show any visible changes in morphology. SVGp12 cells cultured in serum free medium showed reduced confluency and an increase in necrotic cells (I). (J) SVGp12 cells cultured in standard 10 % FBS conditions. Images taken following 48 hrs culture in replacement serum concentrations at x10 magnification.

3.2.3 Immunostaining of U87MG and SVGp12 following serum replacement indicated cells had not undergone an epithelial-mesenchymal transition

Changes in serum type could stimulate changes in cell phenotype due to differences in composition such as growth factors and miRNAs. To determine whether the morphological changes observed in Section 3.2.2 following serum replacement were associated with changes in cell phenotype, cells were immunostained for various markers. Neuronal and epithelial-mesenchymal transition markers were used to determine whether the structures seen were related to de-differentiation and re-differentiation into an alternative cell phenotype and glial markers were also used to determine whether the cell lines had not changed in phenotype. SVGp12 and U87MG cells stained positive for glial fibrillary acidic protein (GFAP), a glial cell marker, and S100B, an astrocyte marker, in all serum conditions (Figure 3.5). U87MG and SVGp12 cell lines cultured in all three serum conditions were negative for the neuronal specific markers (Figure 3.6 and 3.7). In the mesenchymal marker set all immunostaining of cells were negative except for vimentin which was positive for both 10 % glioma patient serum and 10 % FBS control in both SVGp12 and U87MG cultures (Figure 3.8).

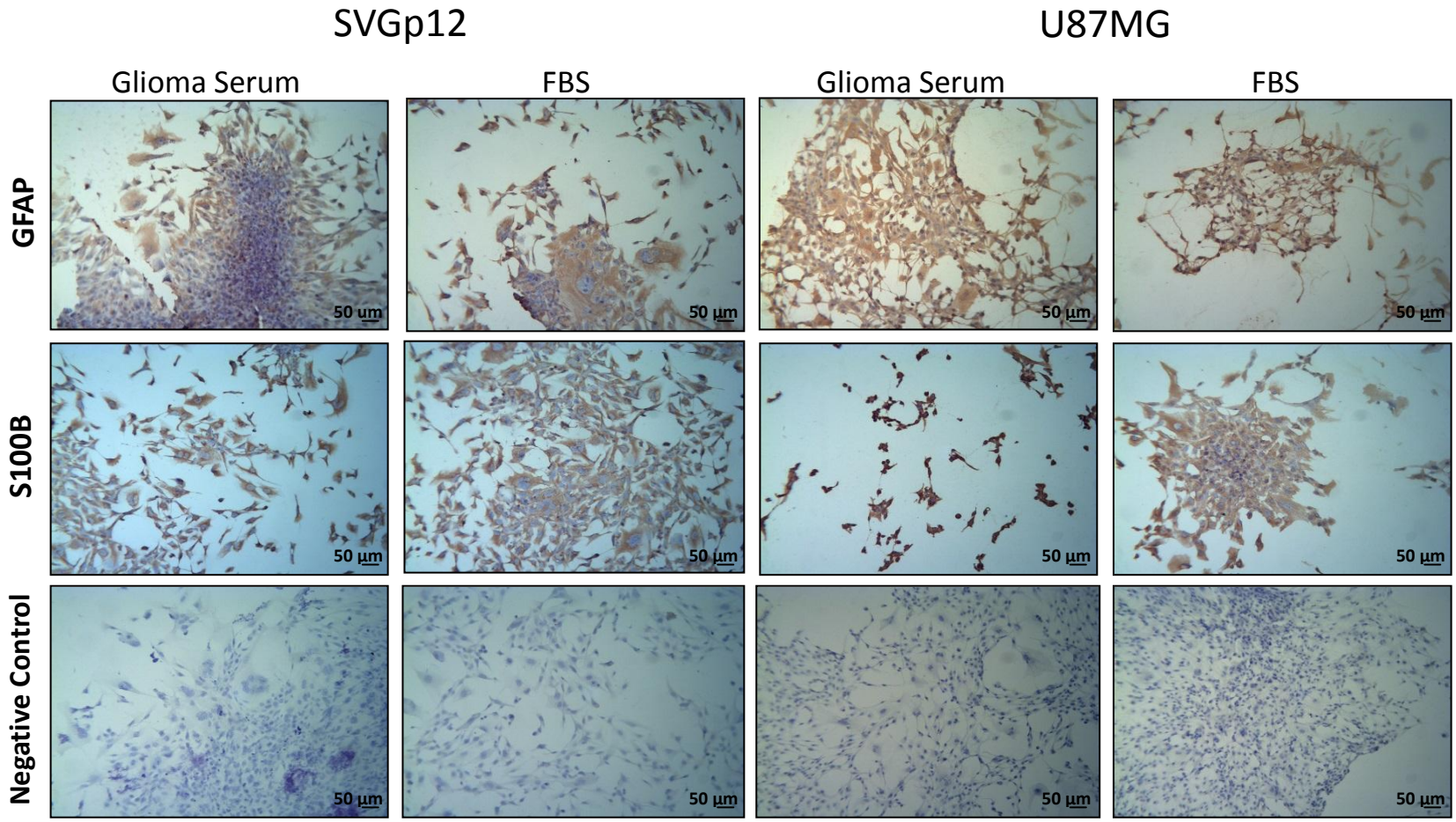


Figure 3.5 Immunostaining of glial markers and negative control in U87MG and SVGp12 cells following serum replacement. U87MG and SVGp12 cells cultured in both glioma patient serum and FBS showed positive staining (indicated by brown colour) for GFAP and S100B and negative staining for the negative control containing no primary antibody. Cells were counterstained with haematoxylin.

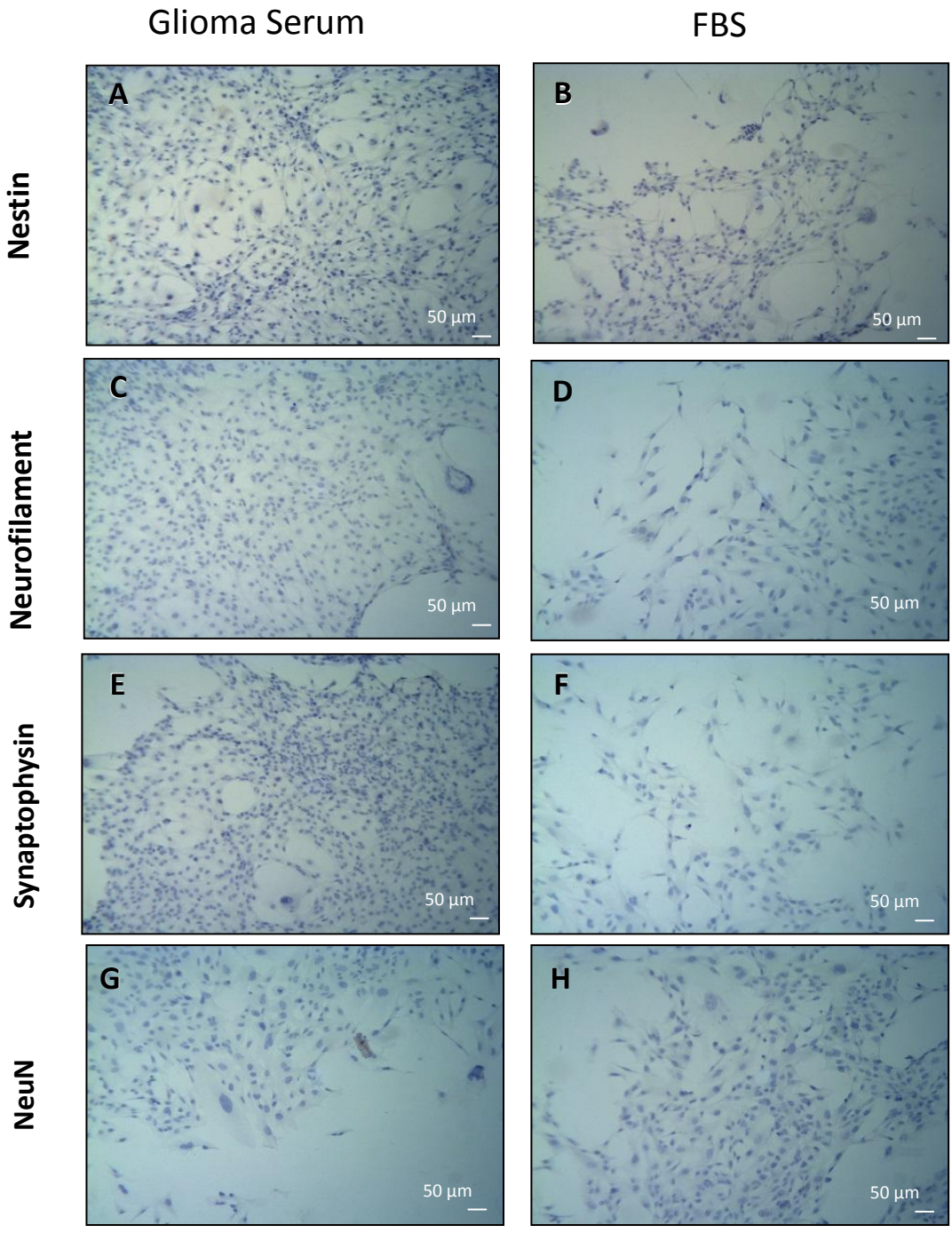


Figure 3.6 Neuronal marker immunostaining of U87MG cells cultured in 10 % glioma patient serum and 10 % FBS. U87MG cells cultured in both glioma patient serum and FBS were negative for all neuronal markers. Positive controls GFAP and S100B (Figure 3.5). Cells were counterstained with haematoxylin.

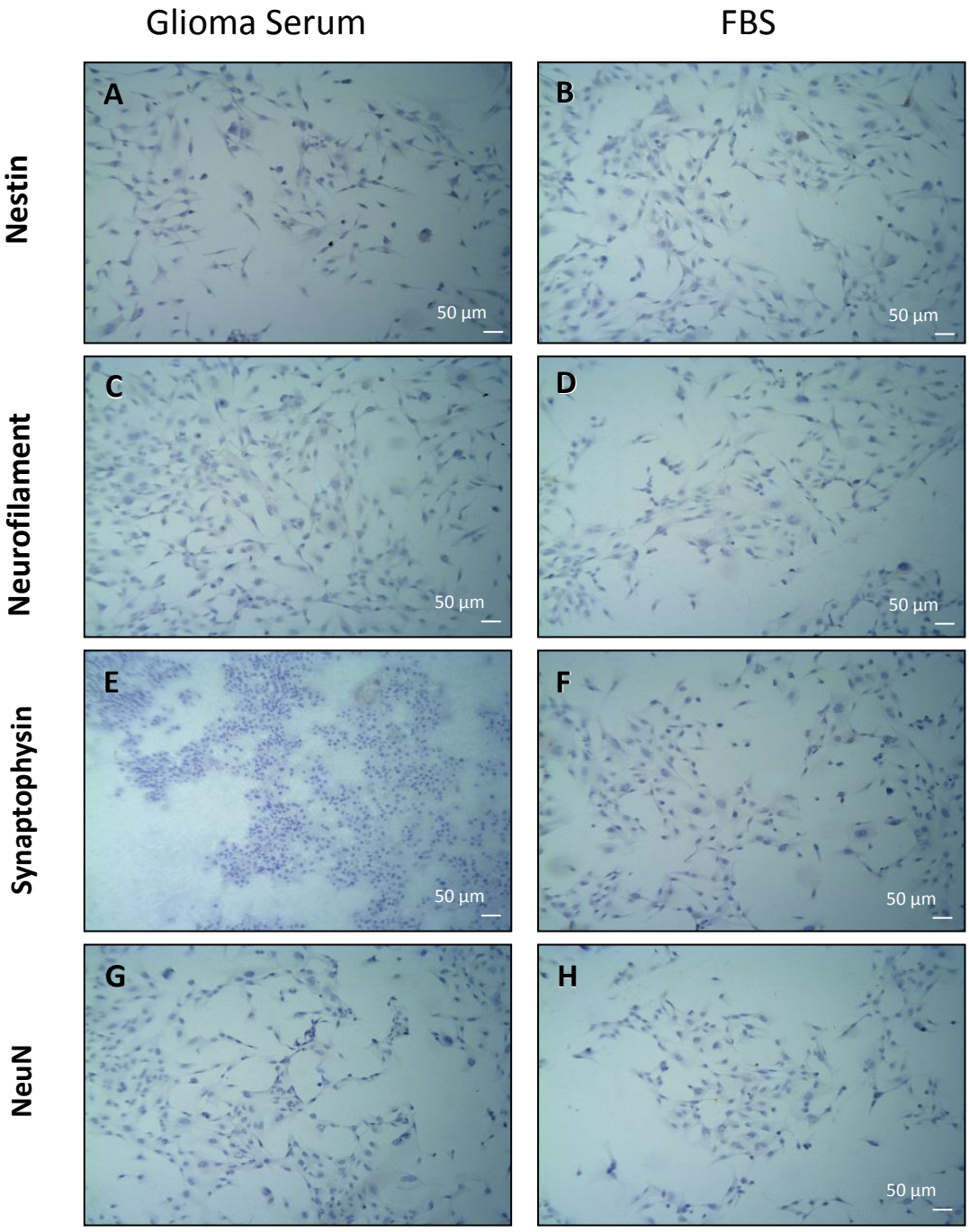


Figure 3.7 Neuronal marker immunostaining of SVGp12 cells cultured in 10 % glioma patient serum and 10 % FBS. SVGp12 cells cultured in both glioma patient serum and FBS were negative for all neuronal markers. Positive controls GFAP and S100B (Figure 3.5). Cells were counterstained with haematoxylin.

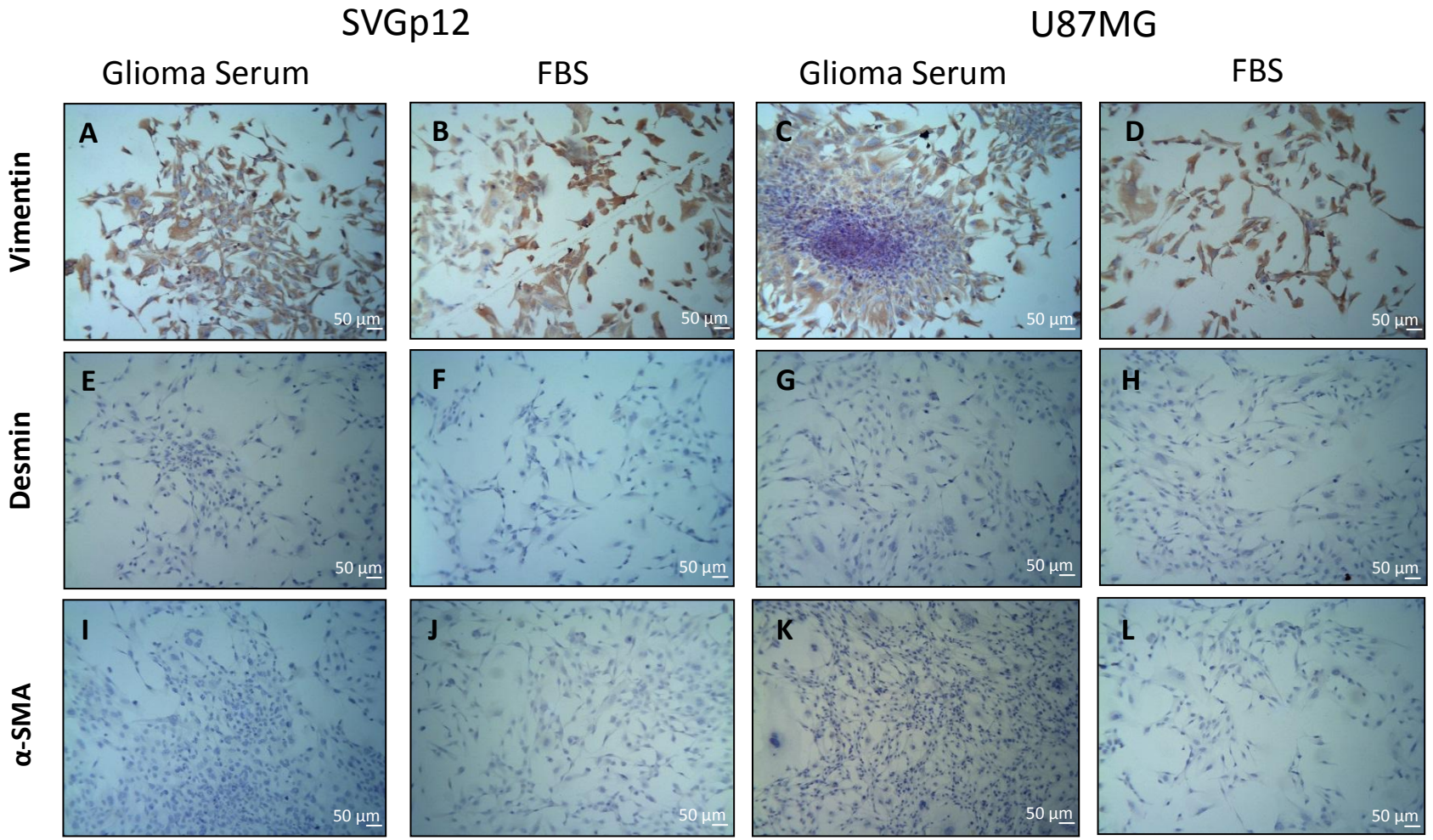


Figure 3.8 Mesenchymal marker immunostaining of U87MG and SVGp12 cells cultured in 10 % glioma patient serum and 10 % FBS. SVGp12 and U87MG cells cultured in both glioma patient serum and FBS stained positive for vimentin. SVGp12 and U87MG cells cultured in both serum conditions were negative for both desmin and α-SMA. Positive controls GFAP and S100B (Figure 3.5).

3.2.4 MicroRNA expression of U87MG in standard culture conditions compared to SVGp12

To elucidate the effect of serum replacement on miRNA expression of U78MG and SVGp12 cell lines, expression of miRNAs in cell lines cultured in standard conditions (10 % FBS) was determined using the miScript brain cancer array panel. MiRNA expression in U87MG cells was measured against SVGp12 cells as a control. MiR-29b-3p showed the greatest increase in fold change of 3.94 in U87MG compared to SVGp12. The remaining three up-regulated miRNAs, miR-9, miR-101 and miR-328, exhibited around a two-fold change (Figure 3.9). Overall there was a greater number of miRNAs with a decrease in expression in the U87MG cell line compared to SVGp12. From the 84 miRNAs within the panel, 28 showed a decrease in expression in the U87MG cell line compared to SVGp12 (Figure 3.10). Comparing the 28 miRNAs with reduced expression, miR-326 and miR-31-5p showed the greatest decrease in fold change of 0.0065 and 0.0244 respectively.

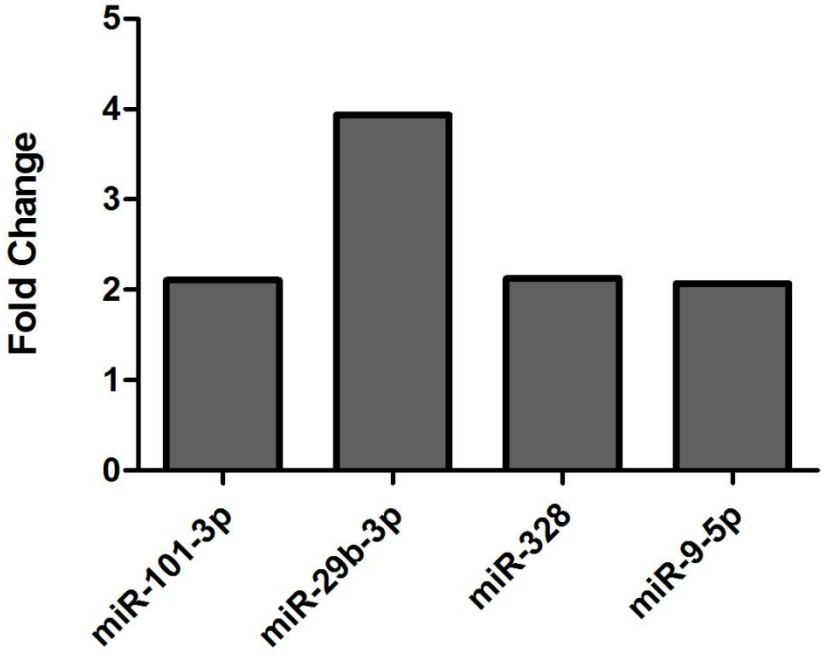


Figure 3.9 Intracellular expression of up-regulated miRNA in U87MG in standard culture conditions. Four miRNAs showed an increase in expression in U87MG cells cultured in 10 % FBS (n=1) compared to SVGp12 cells (n=1). MiR-29b-3p showed the greatest increase in expression. MiR-101-3p, miR-328 and miR-9-5p showed a similar increase in expression in U87MG cells. Data shown as fold change expression compared to SVGp12 set at a standard value of 1.

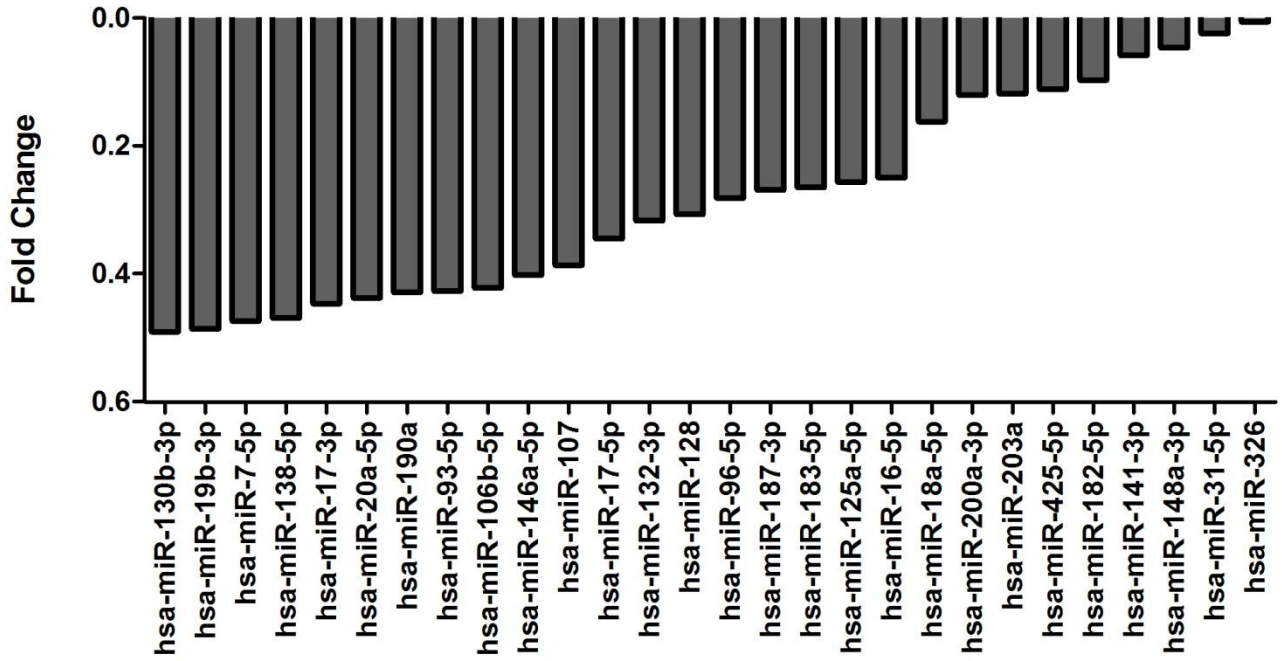


Figure 3.10 Intracellular expression of miRNA with reduced expression in U87MG in standard culture conditions. A total of 28 miRNAs showed reduced expression in U87MG cells cultured in 10 % FBS (n=1) compared to SVGp12 cells (n=1). MiR-326 and miR-31-5p showed the greatest decrease in expression in U87MG cells. Data shown as fold change expression compared to SVGp12 set at a standard value of 1.

3.2.5 MicroRNA expression in U87MG and SVGp12 cells following Serum Replacement

Following analysis of miRNA expression of U87MG and SVGp12 in standard FBS conditions (Section 3.2.4), the four up-regulated miRNAs (miR-101-3p, 29b-3p, 328, 9-5p) and four miRNAs (miR-326, 31-5p, 141, 148a) with the largest magnitude of reduced expression were chosen to determine whether serum replacement would cause a change in intracellular miRNA expression in the cell lines. U87MG and SVGp12 cells were weaned from 10 % FBS onto either 10 % serum from glioma patients or 10 % serum from non-cancerous patients (Section 2.1.7). Cells were cultured for 48 hours in 10 % replacement serum to allow one full cell cycle, then total RNA was extracted and used to analyse miRNA expression. Further to this, extracellular miRNA expression in the spent media of weaned cell lines was compared to those cultured in FBS, to determine whether serum replacement would affect the expression of secreted exosomal miRNAs. The miRNAs with reduced expression, miR-141 and 31-5p, were not detected in any of the serum conditions intracellularly or extracellularly for both U87MG and SVGp12.

From the eight miRNAs chosen for this study, miR-148a alone was found to be expressed in exosomes isolated from the spent media of both SVGp12 and U87MG cell lines (Figure 3.11), for all three serum conditions. Changing the serum in the culture media from 10 % FBS to 10 % glioma patient serum or 10 % control serum did not significantly alter miR-148a secreted into the spent media extracellularly for either U87MG or SVGp12 (Figure 3.11A and B). MiR-148a detection was significantly higher ($p < 0.001$) in the medium of U87MG cells cultured in 10 % glioma serum (Figure 3.11C), 10 % non-cancerous serum (Figure 3.11D), and 10 % FBS ($p < 0.01$)(Figure 3.11E) compared to the media of SVGp12 cells.

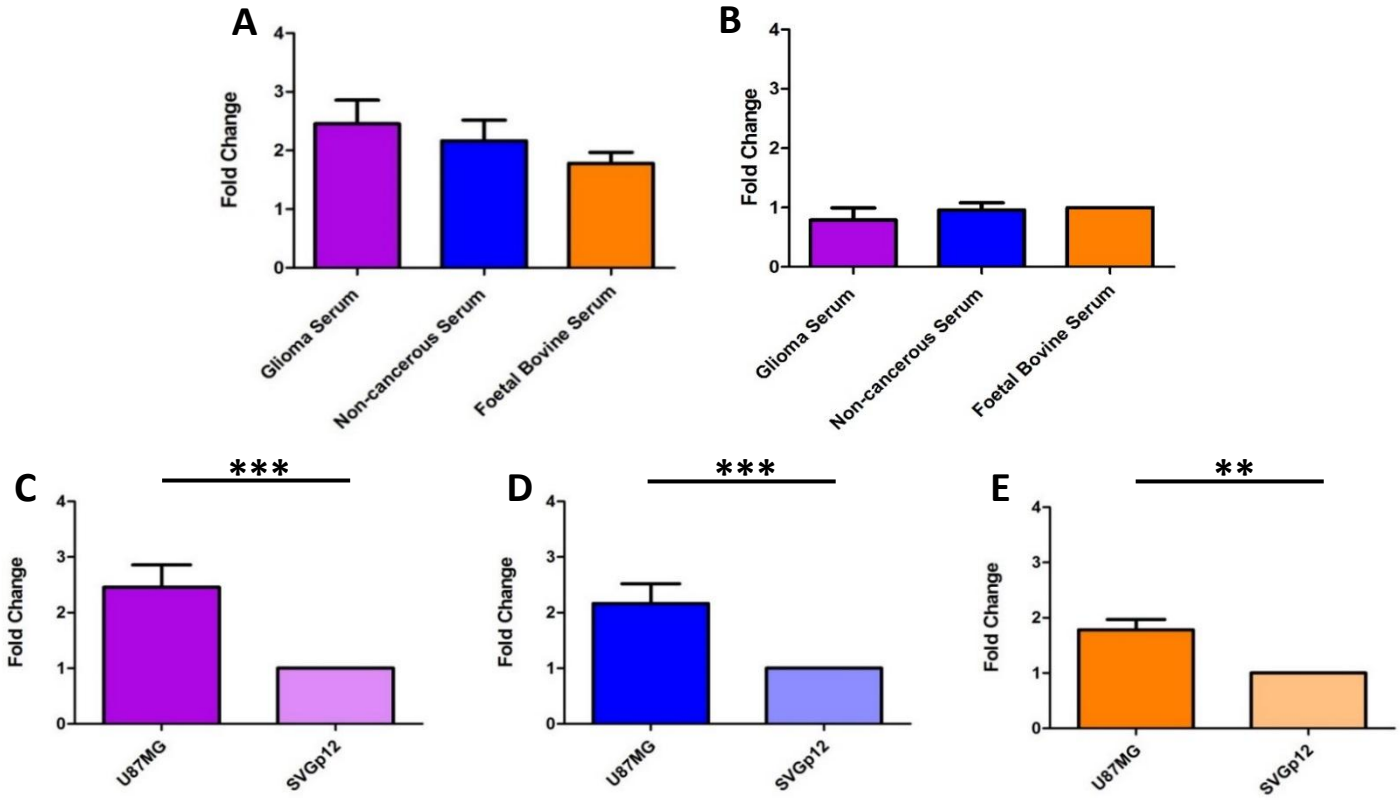


Figure 3.11 Extracellular expression of miR-148a in U87MG cells following serum replacement. A) Comparison of miR-148a expression in U87MG cultured in different serum conditions. No significant difference was observed between the three serum conditions by one-way ANOVA with Tukey *post-hoc* test. B) No significant difference in miR-148a expression was observed in SVGp12 cells cultured in 10 % replacement human serum compared to 10 % FBS by one-way ANOVA with Tukey *post-hoc* test. C) MiR-148a expression in U87MG cells cultured in 10 % glioma patient serum was significantly higher compared to SVGp12 by student's t-test. D) MiR-148a expression in U87MG cells cultured in 10 % non-cancerous human serum was higher compared to SVGp12. E) MiR-148a expression in U87MG cells cultured in 10 % FBS was significantly higher compared to SVGp12.Data shown as mean of triplicate experiments with SD with control SVGp12 expression set to a standard value of 1. Significance between groups indicated by a solid black line, $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

A comparison of intracellular U87MG cells cultured in the three different serum conditions showed a significantly ($p < 0.01$) higher level of expression of miR-148a in U87MG cells cultured in non-cancerous human serum and those cultured in FBS (Figure 3.12A). There was no significant difference in intracellular expression of miR-148a in SVGp12 cells cultured in the three serum conditions (Figure 3.12B). Intracellular miR-148a expression was significantly ($p < 0.01$) reduced in U87MG cells cultured in glioma patient serum and FBS compared to SVGp12 cells cultured in the same conditions (Figure 3.12C and D). There was no significant difference in intracellular miR-148a expression between U87MG and SVGp12 cells cultured in non-glioma patient serum (Figure 3.12D).

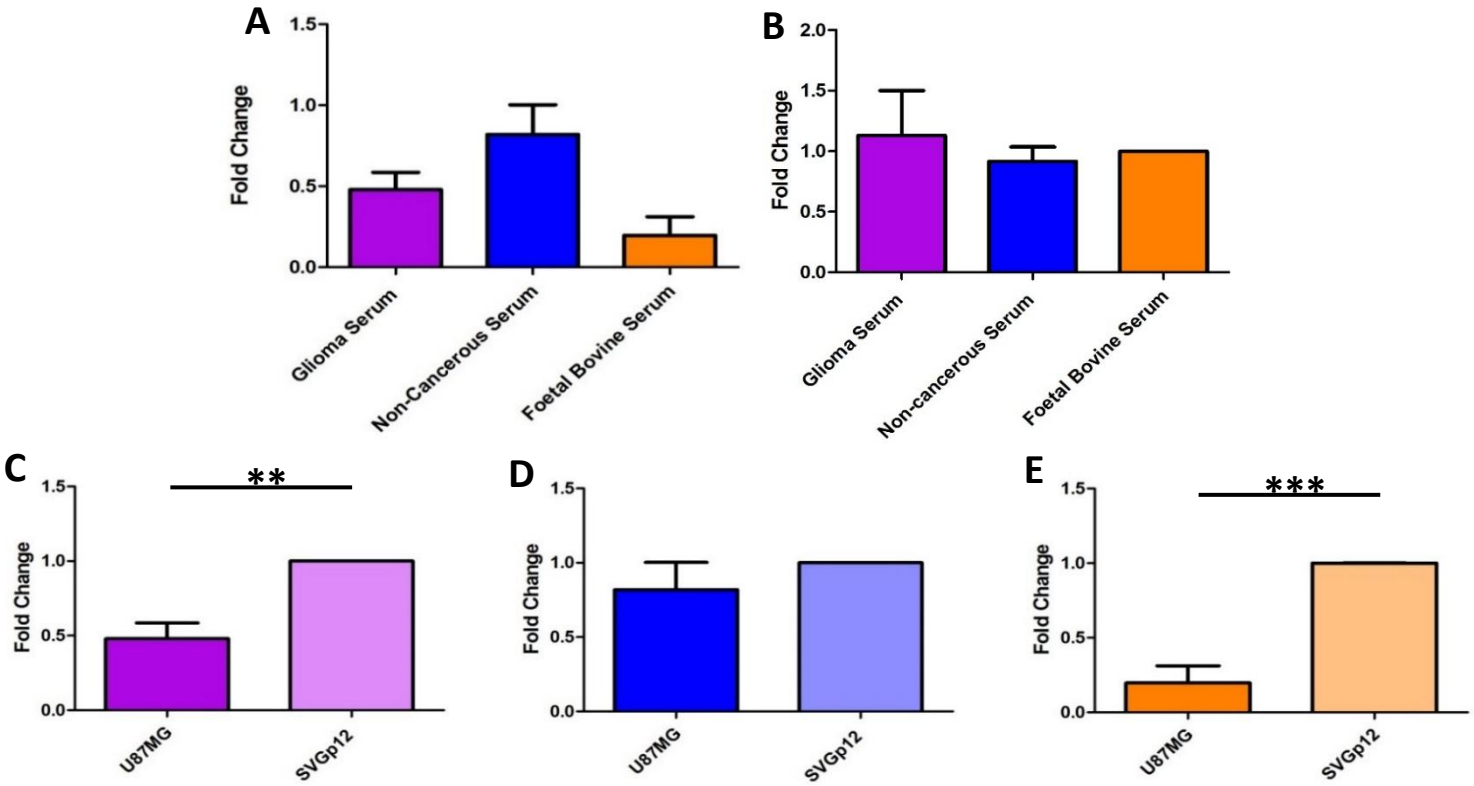


Figure 3.12 Intracellular expression of miR-148a in U87MG cells following serum replacement. A) Comparison of miR-148a expression in U87MG cells cultured in three serum conditions. MiR-148a was significantly higher in U87MG cells cultured in 10 % non-cancerous serum compared to 10 % FBS by one way ANOVA with Tukey *post-hoc* test. B) Expression of miR-148a in SVGp12 cells cultured in varying serum conditions was not significantly different. C) Expression of miR-148a in U87MG cells cultured in 10 % glioma patient serum was reduced compared to SVGp12, student's t-test. D) Expression of miR-148a in U87MG cells cultured in 10 % non-cancerous human serum was not significantly different compared to SVGp12. E) Expression of miR-148a in U87MG cells cultured in 10 % FBS was down-regulated compared to SVGp12. Data shown as mean of triplicate experiments with SD with control SVGp12 values set to a standard value of 1 (A, C-E) and B) control FBS set to a standard value of 1. Significance between groups indicated by a solid black line, $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

MiR-101 was expressed in U87MG cells alone (Figure 3.13). Furthermore, expression of miR-101 was not detected in the spent media of U87MG cells cultured in all three serum conditions. Higher expression of miR-101 in U87MG cells compared to SVGp12 correlated with the data shown in Figure 3.9. MiR-101 was significantly higher (p = 0.0106) in U87MG cells cultured in 10 % glioma patient serum compared to those cultured in FBS. U87MG cells cultured in 10 % non-cancerous human serum also exhibited a significantly higher expression (p = 0.0106) of miR-101 compared to FBS. There was no significant difference in miR-101 expression between U87MG cells cultured in glioma patient serum and non-cancerous human serum.

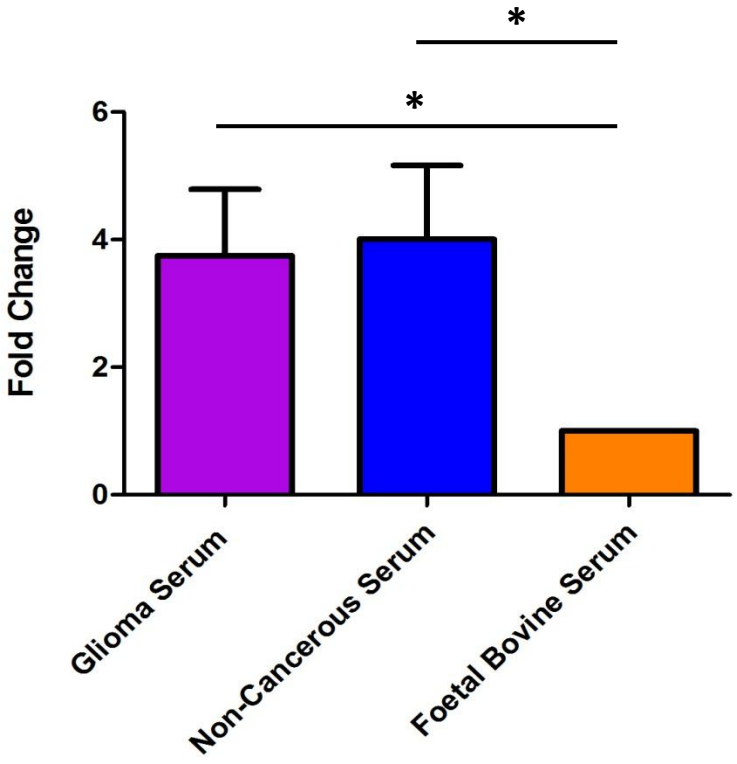


Figure 3.13 Intracellular expression of miR-101 in U87MG cells following serum replacement. Expression of miR-101 in U87MG cells cultured in 10 % glioma patient serum and 10 % non-cancerous human serum was significantly higher compared to U87MG cells cultured in standard 10 % FBS conditions by one way ANOVA with Tukey *post-hoc* test, p<0.05. Data shown as mean of triplicate experiments with SD with 10 % FBS control value set to a standard value of 1. Significance between groups indicated by a solid black line, *p = 0.0106.

Intracellular miR-29b was significantly higher (p < 0.01) in all three serum conditions for U87MG compared to SVGp12 (Figure 3.14B, C and D).

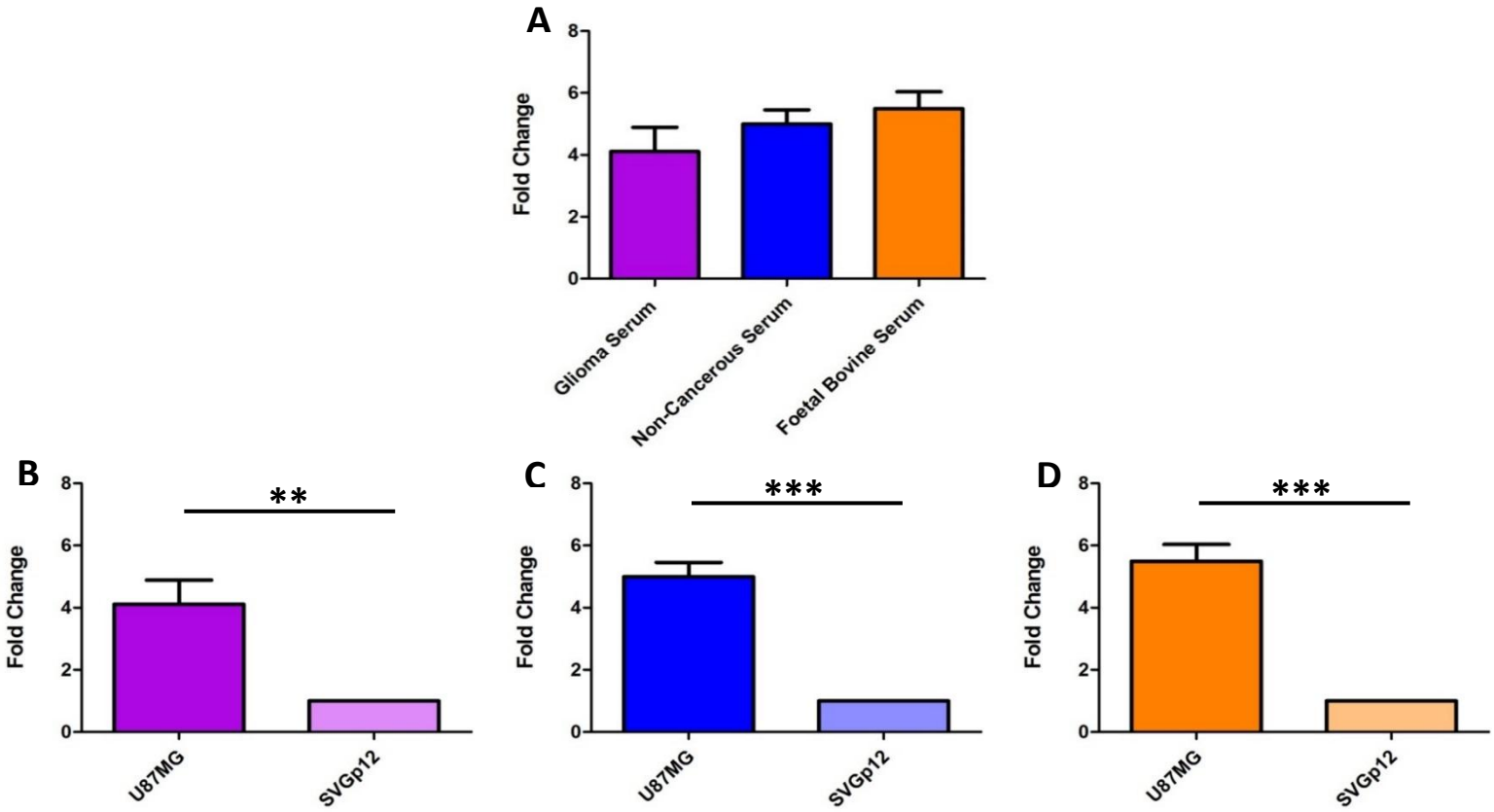


Figure 3.14 Intracellular expression of miR-29b in U87MG cells following serum replacement. A) Comparison of miR-29b in U87MG cells cultured in three serum conditions. There was no significant difference in miR-29b expression in U87MG cells cultured in 10 % human serum or 10 % FBS. B) Expression of miR-29b in U87MG cells cultured in 10 % glioma patient serum was up-regulated compared to SVGp12, student's t-test, p <0.01. C) Expression of miR-29b in U87MG cells cultured in 10 % non-cancerous human serum was up-regulated compared to SVGp12, student's t-test, p < 0.001. D) Expression of miR-29b in U87MG cells cultured in 10 % FBS was up-regulated compared to SVGp12, student's t-test, p < 0.001. Data shown as mean of triplicate experiments with SD with control SVGp12 expression set to a standard value of 1.

MiR-328 was not expressed in SVGp12 cells in any of the three serum conditions but was expressed in U87MG cells (Figure 3.15). Expression was slightly reduced in U87MG cells cultured in 10 % glioma serum compared to the FBS control. U87MG cells cultured in 10 % non-cancerous human serum expressed miR-328 at a slightly higher level than cells cultured in FBS. The changes observed however, were not significantly different ($p > 0.05$).

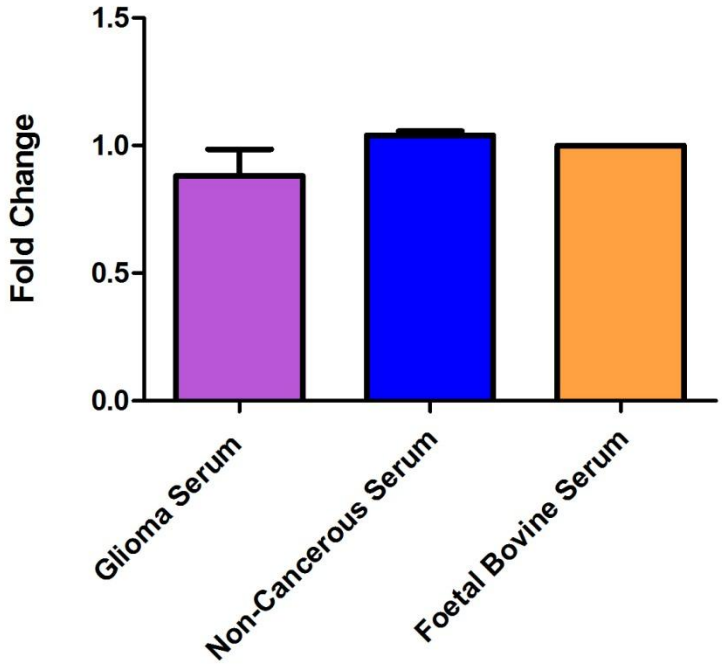


Figure 3.15 Intracellular expression of miR-328 in U87MG cells following serum replacement. Expression of miR-328 in U87MG cells cultured in 10 % glioma patient serum and 10 % non-cancerous human serum was not significantly different compared to U87MG cells cultured in 10 % FBS. Data shown as mean of triplicate experiments with SD with control FBS set to a standard value of 1.

MiR-9 was also expressed in U87MG cells alone and in all three serum conditions miR-9 was not significantly reduced ($p > 0.01$) in U87MG cells cultured in glioma patient serum compared to those cultured in FBS. No significant difference in expression ($p > 0.01$) was observed between U87MG cells cultured in non-cancerous human serum and either FBS or glioma patient serum (Figure 3.16).

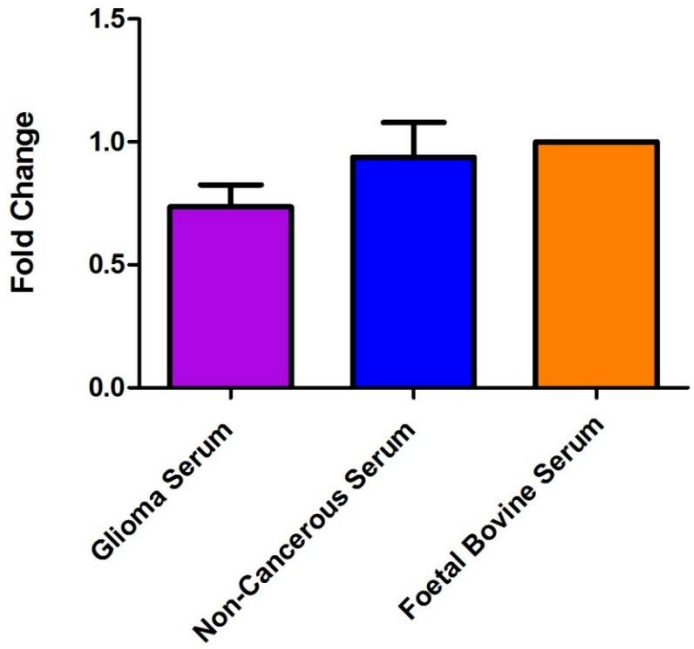


Figure 3.16 Intracellular expression of miR-9 in U87MG cells following serum replacement. Expression of miR-9 in U87MG cells cultured in both 10 % non-cancerous human serum and 10 % glioma patient serum was not significantly different compared to U87MG cells cultured in 10 % FBS $p > 0.01$. Data shown as mean of triplicate experiments with SD with control FBS set to a standard value of 1.

Comparison of the three serum conditions showed that miR-326 was significantly higher ($p < 0.001$) in U87MG cells cultured in non-cancerous human serum compared to U87MG cells cultured in glioma patient serum and FBS (Figure 3.17A). In contrast, miR-326 was not significantly different ($p > 0.01$) in SVGp12 cells grown in glioma serum compared to cells grown in non-cancerous serum (Figure 3.17B). SVGp12 cells grown in either 10 % glioma serum or FBS showed no significant difference in intracellular miR-326 expression than U87MG cells grown in the same conditions ($p > 0.01$) (Figure 3.17C and). Conversely, U87MG cells grown in 10 % control patient serum showed a significantly higher level of intracellular miR-326 expression than SVGp12 ($p < 0.01$).

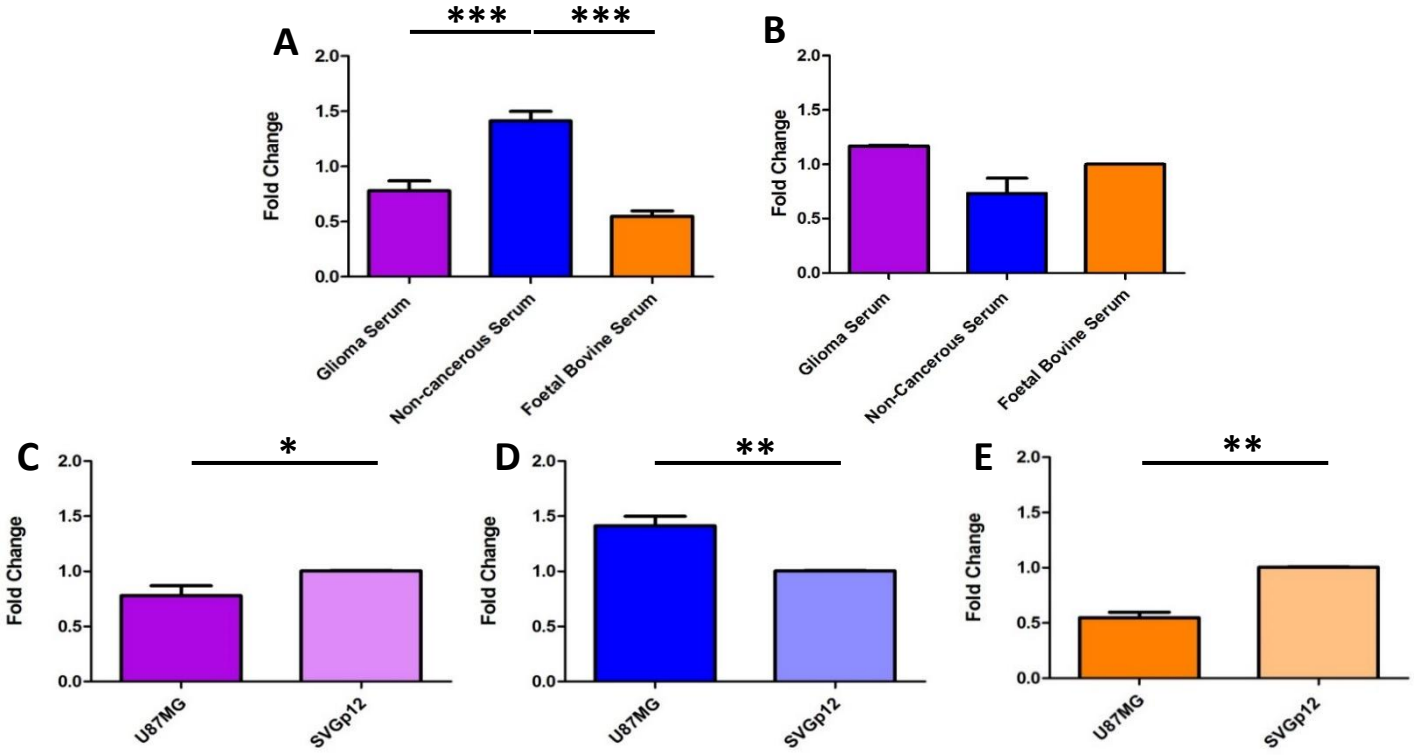


Figure 3.17 Intracellular expression of miR-326 in U87MG cells following serum replacement. A) Comparison of miR-326 expression in U87MG cells cultured in different serum conditions. MiR-326 was significantly increased in U87MG cells cultured in 10 % non-cancerous human serum compared to those cultured in 10 % FBS. U87MG cells cultured in 10 % non-cancerous human serum had a higher expression of miR-326 compared to those cultured in 10 % glioma patient serum. B) Expression of miR-326 in SVGp12 cells grown in varying serum conditions was not significantly different between SVGp12 cells cultured in 10 % glioma serum and 10 % non-cancerous serum. C) Expression of miR-326 in U78MG cells in 10 % glioma patient serum was reduced compared to SVGp12. D) Expression of miR-326 in U87MG cells cultured in 10 % non-cancerous human serum was higher compared to SVGp12. E) Expression of miR-326 in U87MG cultured in 10 % FBS was reduced compared to SVGp12. Data shown as triplicate experiments with SD with control SVGp12 set to a standard value of 1 (A, C-E) and B) control FBS set to a standard value of 1. Significance between groups indicated by solid black line, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.3 Discussion

A human cell culture model for the investigation of miRNA expression has been developed using serum replacement techniques to wean cells off FBS and onto human serum. The differential effects of human serum from both glioma and non-cancer control subjects on the morphology and growth of U87MG and SVGp12 cells suggests a variance in sera compositions. Whilst the precise difference in serum composition is unknown, changes in miRNA expression as identified in previous studies could potentially be a contributing factor to the results observed in this study (Roth et al., 2011, Yang et al., 2013b). Previously, analysis of tumour derived exosomes has identified differences in protein, mRNA and miRNA expression (Xiao et al., 2012). Profiling of melanoma cell line derived exosomes and non-cancerous melanocyte cell line exosomes showed distinct profiles of differentially expressed proteins and mRNAs as well as tumour-specific oncogenic miRNAs (Xiao et al., 2012). The proteins and RNA present within melanoma exosomes were found to play a role in cell migration, invasion and proliferation (Xiao et al., 2012). The cellular uptake of exosomes of different protein and RNA composition present in non-cancerous and cancerous serum could therefore result in differences in cell growth and phenotype as observed in this study.

In this study, the culture of U87MG and SVGp12 cells in sera from both cancerous and non-cancerous subjects resulted in an increase in cell number compared to standard FBS. The presence of exosomes within the replacement serum could have resulted in the increase in cell number. The application of glioblastoma exosomes to U87MG cells has previously been shown to increase their proliferative potential (Skog et al., 2008). The application of exosomes to cell culture in increasing concentrations has also shown a dose-dependent increase in proliferation and glycolytic activity (Graner, 2011). In the current study however, cells were not cultured with human derived exosomes alone but with whole replacement serum containing growth factors and signalling molecules which could also cause an increase in cell number. To determine whether the increase in cell number was a result of exosomes U87MG and SVGp12 cells could be cultured with exosomes, enriched from cancerous and non-cancerous serum and cell numbers compared to U87MG and SVGp12 cells cultured in whole replacement serum and standard FBS.

The changes in morphology observed following serum replacement could have been a result of transdifferentiation of cells into a different phenotype. Initial investigations were therefore performed to determine whether the changes were due to the expression of neuronal markers. Immunohistochemical staining for neuronal markers however was negative for both U87MG and SVGp12 in all three serum conditions. This suggested that the acquisition of a neuronal phenotype had not occurred following serum replacement.

In glioma, including glioblastoma, cells undergo an epithelial-mesenchymal transition (EMT) to facilitate tumour progression and metastasis. Tumour cells undergoing EMT undergo both molecular reprogramming and morphological changes to acquire invasive characteristics which allow them to alter the microenvironment, enter the stroma and promote metastasis (Iwatsuki et al., 2010). EMT is initially characterised by the loss of endothelial markers such as E-cadherin and the acquisition of mesenchymal markers such as vimentin, through alterations in signalling pathways which regulate these proteins. E-cadherin is an epithelial-specific transmembrane glycoprotein which forms tight junctions with neighbouring cells, reduced expression of E-cadherin causes the loss of cell-cell adhesion (Iwatsuki et al., 2010). Clinically, the loss of E-cadherin is often associated with an aggressive phenotype and poor prognosis (Gregory et al., 2008). Following the induction of mesenchymal marker expression, cells undergo cytoskeletal remodelling and subsequent changes in cell-matrix adhesion through activation of proteolytic enzymes such as MMPs (Iwatsuki et al., 2010).

Research has also identified a role for miRNAs in EMT miRNA microarray profiling has identified the miR-200 family and miR-205 as regulators of EMT. These miRNAs repress EMT by targeting critical signalling pathways and inhibitors of E-cadherin, ZEB1 and ZEB2 (Gregory et al., 2008). Down-regulation of these miRNAs in cancer promotes EMT and subsequent invasion (Gregory et al., 2008). Following transition to a mesenchymal phenotype, cells display a spindle-shaped morphology and have increased migratory ability (Iwatsuki et al., 2010).

Following serum replacement and culture in 10 % glioma serum, U87MG cells were observed to have spindle-shape morphology consistent with that observed following EMT. This morphology however was also observed in U87MG cells cultured in 10 %

non-cancerous serum suggesting that factors within the serum which caused this alteration may not be glioblastoma specific and not a result of EMT. Further to this, staining for EMT markers was negative except for vimentin which stained positive for both cell lines cultured in both glioma serum and FBS. Vimentin is an intermediate filament expressed in cells (Schiffer et al., 1986) that is up-regulated in EMT (Lee et al., 2006). The strength of staining for the vimentin marker in both cell lines did not appear to be increased in the U87MG cells compared to those cultured in glioma serum compared to FBS and the SVGp12 cell line cultured in both serum conditions. Along with the negative staining of desmin and α-SMA EMT markers, this suggests that the U87MG cells cultured in glioma serum had not undergone EMT.

Both U87MG and SVGp12 stained positive for the glial marker GFAP and the astrocyte marker S100B. GFAP is the main intermediate filament in astrocytes which modulates astrocyte motility and shape (Eng et al., 2000). S100B is a protein of the S100-calmodulin-troponin family and is an astrocyte specific marker (Adami et al., 2001). Positive staining for the glial and astrocyte cell markers again supported the conclusion that the cells had not undergone EMT.

Cells undergo changes in shape and therefore morphology by rearrangement of the cytoskeleton changes (Bissell et al., 2003), biochemical changes and changes in gene expression (Kenny et al., 2007). The culture of U87MG cells in both cancerous and non-cancerous human serum caused changes in morphology, culture of SVGp12 cells in the same conditions however, did not result in any clear changes in morphology and in addition, immunostaining confirmed retention of a glial phenotype. This suggests a cell type specific response to changes in serum conditions which may be a result of differences in gene and protein expression between the two cell lines and not transdifferentiation into a new cell phenotype.

In comparison to replacement of serum type, the effect of serum-free conditions on cell line morphology U87MG and SVGp12 cells was also determined. Cells were cultured in decreasing concentrations of FBS and increasing concentrations of serum free medium. The culture of both cell lines in serum-free medium resulted in the formation of spheroids, a visual reduction in cell number and an increase in necrotic cells. There were no apparent similarities in morphology between U87MG and SVGp12

cells cultured in serum-free medium compared to those cultured in both cancerous and non-cancerous human serum. When cultured in serum free medium both cell lines showed an increase in necrotic cells and a reduction in cell number, this suggested that the cell may have been weaned too quickly to adjust to the stress of the new serum condition rather than an effect of the serum free medium.

To determine whether the culture of U87MG and SVGp12 in human serum caused a change in miRNA expression, miRNA profiles of U87MG cells compared to SVGp12 cells both grown in standard 10 % FBS serum conditions were determined. U87MG cells in standard culture conditions showed reduced expression of 28 miRNAs and increased expression of four miRNAs compared to non-cancerous SVGp12 cells. The general decrease in miRNA expression is similar to the global decrease in miRNA expression observed in tumour tissues (Calin and Croce, 2006). MiR-326 and 31-5p showed the greatest decrease in expression in U87MG cell lines compared to SVGp12. Out of the 28 miRNAs reduced in U87MG cells cultured in 10 % FBS, 16 are expressed at a lower level in glioblastoma tissue (Table 3.1) and 12 are increased (Table 3.2). MiR-190a expression, targets and role in glioma has not previously been studied. MiR-187 altered expression has been identified, however, targets and function in glioma has not yet been elucidated. MiR-130b has been identified to have a role in the regulation of metastasis, however the targets of miR-130b have not yet been validated.

Table 3.1 Expression of miRNA down-regulated in both U87MG cells and glioblastoma tissue

MicroRNA	Expression in GBM	Targets	Role	Reference
miR-107	Down	VEGF, Notch 2	Migration, Invasion	(Singh et al., 2012, Chen et al., 2013)
miR-125a	Down	ERBB2, PDPN	Invasion	(Henriksen et al., 2014a, Cortez et al., 2010)
miR-128	Down	E2F3a	Proliferation	(Zhang et al., 2009)
miR-132	Down	CDKN1A	Gliomagenesis	(Singh et al., 2012)
miR-138	Down	IGF1R, ROCK2	Proliferation, apoptosis	(Wang et al., 2012, Singh et al., 2012)
miR-146a	Down	EGFR, Notch 1	Glioma growth and migration	(Mei et al., 2011)
miR-16	Down	BCL2	Glioma growth and invasion	(Yang et al., 2014)
miR-18a	Down	Smad3	Migration, radiation resistance	(Fox et al., 2013)
miR-200a	Down	B-catenin	Cell growth and survival	(Su et al., 2012)
miR-203a	Down	Robo1	Migration	(Dontula et al., 2013)
miR-31	Down	Radixin	Invasion and migration	(Hua et al., 2012a)
miR-326	Down	PKM2, Notch 1/2	Metabolism, proliferation	(Singh et al., 2012)
miR-7	Down	EGFR	Invasion	(Kefas et al., 2008)
miR-187	Down	-	-	(Wang et al., 2012)
miR-425	Down	Notch1/2	Proliferation, radioresistance, 'stemness'	(Singh et al., 2012)

Table 3.2 Expression of miRNA down-regulated in U87MG cells and up-regulated in glioblastoma tissue

MicroRNA	Expression in GBM	Targets	Role	Reference

miR-183	Up	EGR1	Migration	(Sarver et al., 2010)
miR-106b	Up	RBL2	Proliferation	(Zhang et al., 2013)
miR-130b	Up	-	Metastasis	(Su et al., 2010)
miR-141	Up	PTEN, TGFB2	Glioma growth, invasion	(Singh et al., 2012, Koul, 2008)
miR-148a	Up	MIG6, BIM	Apoptosis	(Kim et al., 2014)
miR-17-5p	Up	PTEN	Invasion, migration	(Li and Yang, 2013)
miR-17-3p	Up	MDM2	Proliferation and drug resistance	(Li and Yang, 2013)
miR-182	Up	CYLD, TNIP1, OPTN, USP15	Invasion, angiogenesis	(Song et al., 2012)
miR-19b	Up	PTEN	Gliomagenesis	(Jia et al., 2013)
miR-20a	Up	E2F1/2/3, PTEN, NRAS	Apoptosis, proliferation	(Sylvestre et al., 2007, Singh et al., 2012)
miR-93	Up	Integrin-β8	Glioma growth, angiogenesis	(Fang et al., 2011)
miR-96	Up	HBP1	Proliferation	(Yan et al., 2014)

Three of the four miRNAs with increased expression in U87MG cells cultured in standard FBS conditions have been found to show reduced expression in glioblastoma tissue. The four miRNAs with increased expression in U87MG cells were miR-101-3p, 29b-3p, miR-328 and miR-9-5p. MiR-101 expression is reduced in glioblastoma tissue which subsequently promotes proliferation, migration and angiogenesis due to the overexpression of the miR-101 target EZH2 (Smits et al., 2010). MiR-29b is also down-regulated in glioblastoma tissue and plays a role in suppressing invasion by targeting podoplanin membrane sialo-glycoprotein (PDPN) (Cortez et al., 2010). MiR-29b also targets Cdc42 a GTPase which regulates cell morphology and migration signalling

pathways (Cortez et al., 2010) and can induce apoptosis by up-regulating p53 (Park et al., 2009). MiR-328 expression is also reduced in glioblastoma tissue and plays a role in cell cycle progression, high expression of miR-328 in U87MG cells has been shown to suppress proliferation (Wu et al., 2012). MiR-9 has been found to be highly expressed in glioblastoma tissue and suppresses the mesenchymal differentiation of GBM cells as well as promoting chemoresistance to TMZ by inducing the expression of the drug efflux transporter, p-glycoprotein (Munoz et al., 2013).

Differences in expression between the U87MG cell line and glioblastoma may be a result of culture artefacts, such as mutations which occur as a result of establishment and long term culture of cells, therefore acquired mutations may cause an alteration in miRNA expression not representative of glioblastoma. Genomic analysis of established glioma cell lines including U87MG identified recurrent aberrations and global gene expression clusters not represented in primary gliomas, suggesting that established glioma cell lines, including U87MG, are poorly representative of primary gliomas (Li et al., 2008). Although miRNA expression was not determined by Li *et al.*, changes in gene expression will most likely affect miRNA expression and therefore the data observed in this study. Conversely, the use of one U87MG and SVGp12 sample may have affected the data observed as the changes in miRNA expression observed in this study may be specific to these samples.

Following identification of miRNAs with altered expression in U87MG cells cultured in FBS, the effect of serum replacement on the expression of selected miRNAs identified was investigated. MiR-148a alone was detected extracellularly in exosomes isolated from the spent media. The presence of this single miRNA out of the 8 chosen may be due to the selective packaging of exosomes (Chen et al., 2012). MiR-148a plays a role in a number of glioblastoma functions including cell growth, survival, migration and invasion (Kim et al., 2014). MiR-148 targets include MIG6 which regulates EGFR, and BIM which regulates apoptosis (Kim et al., 2014). The extracellular presence of miR-148a may be due to its migratory and invasive promoting properties, as uptake of exosomal miR-148a by recipient cells may result in recruitment of neighbouring cells in the tumour microenvironment to promote invasion (Skog et al., 2008). Comparison of intracellular and extracellular miR-148a expression showed that expression of this miRNA was higher in the medium than in the cell. Again, this may be due to selective

exosomal packaging of miRNAs. Expression of miR-148a was higher in U87MG cells cultured in non-cancerous human serum compared to glioma serum.

MiR-148a exhibited a higher intracellular expression in U87MG cells cultured in glioma serum and non-cancerous serum. This suggests that the culture of U87MG cells in human serum caused an increase in expression of this miRNA. Intracellular expression of miR-148a was higher in U87MG cells cultured in non-cancerous human serum compared to those cultured in glioma serum and this trend was also observed in the extracellular expression of miR-148a. The high expression of miR-148a in U87MG cells cultured in both cancerous and non-cancerous serum suggests factors in human serum which promote the up-regulation of this miRNA. The higher expression of miR-148a in U87MG cells cultured in non-cancerous serum suggests that the components which cause this up-regulation are present at a higher level in non-cancerous serum compared to glioma serum.

Although miR-148a is a tumour promoting miRNA up-regulated in glioblastoma tissue, U87MG cells cultured in FBS showed a reduced expression of miR-148 compared to SVGp12 determined by both the miScript analysis and the subsequent serum weaning study. This again highlights the difference in gene and protein expression observed between the U87MG cell line and glioblastoma tissue (Li et al., 2008).

Out of the eight miRNAs, three were not detected in the SVGp12 cells in any of the culture conditions and miR-141 and 31-5p were not detected in any of the serum conditions for both cell lines. This may be due to the expression of these miRNAs being below the limit of detection. The detection of these miRNAs in U87MG cells suggests that these miRNAs may be expressed at a higher level than in SVGp12 cells.

Expression of miR-101 was not significantly different in U87MG cells cultured in both glioma patient serum and non-cancerous human serum compared to those cultured in FBS. This suggests that factors present in both cancerous and non-cancerous human serum do not affect the expression of miR-101.

The intracellular expression of miR-29b and 328 was not significantly different between the three culture conditions suggesting that factors within the glioma and non-cancerous serum do not affect the expression of these miRNAs or the transcription factors which may target them and regulators of their expression.

Furthermore miR-328 was not detected in SVGp12 cells and expression of miR-29b was up-regulated in all three serum conditions in U87MG compared to SVGp12, suggesting that both the miRNAs are up-regulated in U87MG cells however this up-regulation is not dependent on serum type.

Intracellular expression of miR-9 was highest in U87MG cells cultured in 10 % FBS and was not significantly different in U87MG cells cultured in glioma patient serum compared to FBS. MiR-9 however, has been previously found to be highly expressed in glioblastoma (Malzkorn et al., 2010). MiR-9 inhibits proliferation of glioma cells by targeting CREB and promotes migration by targeting NF1 (Tan et al., 2012a).

Expression of intracellular miR-326 was not significantly different in U87MG cells cultured in 10 % glioma serum and FBS compared to SVGp12. MiR-326 is a tumour suppressor, the down-regulation of which contributes to tumourigenesis and invasion in glioma (Wang et al., 2013). The culture of U87MG cells in non-cancerous human serum had no significant effect on the expression of miR-326. Down-regulation of miR-326 is believed to be as a result of decreased expression of its host gene, *Arrestin β1* (Jiang et al., 2014). MiRNA expression can be down-regulated by transcriptional repression by various transcription factors, therefore the up-regulation of transcription factors in glioma serum compared to non-cancerous human serum may have resulted in the down-regulation observed in U87MG cells cultured in glioma serum and the up-regulation observed in those cultured in non-cancerous serum. Conversely, the presence of proteins in the non-cancerous human serum which may inhibit a miR-326 repressor may have also resulted in the observed up-regulation in U87MG cells cultured in this serum type. MiR-326 has been found to be up-regulated following the knockdown of Notch-1 (Kefas et al., 2009). Furthermore, miR-326 is part of a regulatory feedback loop, in which miR-326 inhibits the Notch pathway and in turn is inhibited itself by Notch (Kefas et al., 2009). Comparison of U87MG miR-326 expression in the three serum conditions showed a significant up-regulation in non-cancerous human serum compared to both glioma patient serum and FBS. MiR-326 was also significantly higher in U87MG cells cultured in glioma patient serum compared to those cultured in FBS.

Analysis of the effects of serum replacement on U87MG and SVGp12 cell lines showed that the culture of these cell lines in both cancerous and non-cancerous human serum had cell growth and morphological effects. Initial studies identifying miRNA expression of cell lines in standard culture conditions showed differences in miRNA expression between U87MG and SVGp12. Cell culture in human serum caused changes in miRNA expression suggesting differences in composition between human and bovine serum. Furthermore changes in miRNA expression were observed in cells cultured in non-cancerous and cancerous serum. The intracellular miRNA expression of cell lines observed in this study was different to that of glioblastoma serum therefore caution should be exercised when extrapolating miRNA data from *in vitro* cell culture, and all components should ideally be defined to accurately determine the source of variation.

Overall, the data collected within this study suggests that the composition of serum is different between patients with and without glioblastoma and that serum can alter miRNA expression of cells in culture. The identification of differences in miRNA could be used for biomarkers for diagnosis of glioblastoma. Further studies were performed to determine whether serum miRNA expression differed between glioblastoma patients and control patients and whether these differences could be used as biomarkers.

4. Identification of Serum MiRNA Biomarkers

4.1 Introduction

MiRNAs are present in the serum of both healthy and diseased individuals. The expression levels of these miRNAs can alter during disease, therefore the identification of miRNA which alter in a disease specific manner could be utilised as biomarkers (Chen et al., 2008). Serum miRNA biomarkers would be particularly useful for diagnosis, prognosis and predicting response to treatment for glioma as they provide a relatively non-invasive test and would require only a blood sample in comparison to the current diagnostic method of surgical brain biopsy. The detection of miRNA in serum samples can be performed by techniques such as SOLEXA sequencing (Ji et al., 2014), qRT-PCR (Kroh et al., 2010) and microarray (Lodes et al., 2009). These techniques are sensitive and can detect low copy numbers of miRNA (Chen et al., 2005).

Data from Chapter 3 (Section 3.2.5) showed that immortalised cell lines U87MG and SVGp12 cultured in either human serum from glioblastoma patients or individuals without glioblastoma differed in the morphology, growth and expression of miRNA. One of the factors which differ between cancerous and non-cancerous serum is the miRNA profile (Chen et al., 2008). Changes in miRNA expression in the serum of glioblastoma patients have already been identified and therefore have the potential to be used as biomarkers for this disease (Yang et al., 2013b). The aim of this chapter was to identify miRNA with altered expression in the serum of glioblastoma patients and investigate the use of miRNAs as diagnostic and prognostic biomarkers. The potential of altered miRNA expression in glioblastoma patient serum for prognostic information was determined utilising survival data of the patients used in this study.

The attractiveness of miRNAs as circulatory biomarkers has led a number of studies to identify serum miRNA signatures which could be used for glioblastoma detection (Baraniskin et al., 2012, Chen et al., 2008, Noerholm et al., 2012), however, few of these studies have investigated the effects of variables such as age and gender on the expression profile of glioblastoma patients. Recently variables such as age and gender were shown to affect serum expression of some miRNAs in non-cancerous individuals (Sawada et al., 2014). As shown in Section 1.1.2, epidemiological data indicate that the

incidence of glioblastoma differs depending on the gender and age of a patient. The incidence of glioma is higher in males compared to females and increases with age (Crocetti et al., 2012) suggesting differences in the molecular characteristics of glioblastomas between individuals which could include miRNA expression. This study therefore aimed to not only identify a serum miRNA profile for glioblastoma patients but to also investigate the effect of age and gender on miRNA expression to provide a more accurate biomarker panel for glioblastoma.

4.2 Results

4.2.1 MiScript MicroRNA Expression in Serum Obtained from Glioblastoma Patients Aged between 20 and 39 Years.

In order to select miRNA biomarkers for glioblastoma, initial studies were performed to determine the miRNA profile of serum from glioblastoma patients analysed in gender and age groups selected based on those used by Crocetti *et al*., (2012). Serum expression profiles were elucidated using the miScript brain cancer array and analysed using the online PCR array tool. MiRNAs were scored either A, B or C by the online tool from the average Ct value, where C was a miRNA whose calculated fold change may not be representative of the actual fold change. These data were used to identify dysregulated miRNAs which could be further studied for use as biomarkers. Serum samples from 18 glioblastoma patients and 18 control patients, three male and three female samples for all three age groups, were individually analysed in matched groups in order to identify age and gender specific miRNA expression.

In the 20-39 glioblastoma age group, eight miRNAs exhibited altered expression in all three serum samples for both males and females compared to age matched controls (Table 4.1). Comparison of the eight miRNAs identified five miRNAs with a similar fold change in expression for both genders with the exception of miR-16-5p, 15b-5p and 451a. MiR-16-5p had a 3.5 fold increase in expression in male samples compared to a 15.3 fold up-regulation in female samples. MiR-15b-5p showed a 2.76 fold increase in expression in female patient serum and a 4.17 fold increase in male patient serum. MiR-486-5p showed a 5.1 fold increase in expression in the male samples however a reduction in expression was observed in the female samples. MiR-451a showed a 4.5 fold increase in expression in male samples whereas female samples showed a 39.7 fold increase.

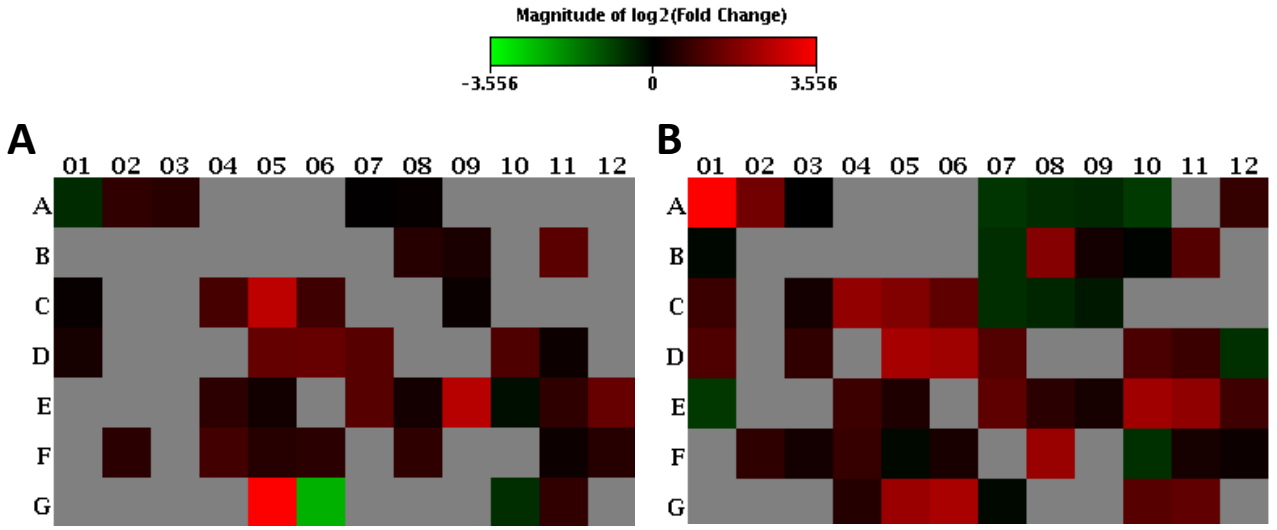
Analysis of serum samples obtained from glioblastoma patients aged between 20 and 39 years identified 13 miRNAs with an increase in expression in all three samples tested in the female group (Figure 4.1A). MiR-451a exhibited the highest fold change increase in expression of 39.7 fold. Analysis of miRNA expression in the serum of male glioblastoma patients aged between 20 and 39 years identified 17 miRNAs with an increase in

expression, but no miRNAs were observed to be reduced. Let-7b-5p exhibited the greatest increase in expression in the male 20-39 age group compared to matched controls (Figure 4.1B).

Three miRNAs showed an increase in expression in the sera of female patients only, miR-29c-3p, 27a-3p and 24-3p (Figure 4.2A). The expression of six miRNAs were increased in the sera of male 20-39 patients alone; let-7-5p, miR-25-3p, 26a-5p, 92a-3p, 144-3p and 320a (Figure 4.2B).

Table 4.1 MiRNAs dysregulated in the serum of both male and female glioblastoma patients aged between 20 and 39 years compared to age and sex matched controls.

	Female		Male	
MiRNA	**Average Fold Change**	**SD**	**Average Fold Change**	**SD**
miR-15b-5p	2.76	1.09	4.17	0.42
miR-16-5p	15.30	6.08	3.50	0.95
miR-17-5p	2.47	2.47	2.46	0.58
miR-19a-3p	4.38	0.86	4.71	0.72
miR-23a-3p	3.43	0.32	2.50	0.29
miR-148a-3p	3.68	1.23	2.25	0.25
miR-191-5p	4.26	0.89	5.07	0.37
miR-451a	39.70	19.73	4.50	0.82



	1	2	3	4	5	6	7	8	9	10	11	12
A	Let-7b-5p	101-3p	106b-5p	107	10b-5p	124-3p	125a-5p	125b-5p	127-5p	128-3p	129-5p	130a-3p
B	130b-3p	132-3p	133a-3p	133b	137	138-5p	141-3p	144-3p	146a-5p	146b-5p	148a-3p	149-5p
C	150-5p	153-3p	15a-5p	15b-5p	16-5p	17-5p	17-3p	181a-5p	181b-5p	182-5p	183-5p	184
D	185-5p	187-3p	18a-5p	190a-5p	191-5p	19a-3p	19b-3p	200a-3p	203a-3p	20a-5p	21-5p	210-3p
E	216a-5p	217	218-5p	221-3p	222-3p	222-5p	23a-3p	23b-3p	24-3p	25-3p	26a-5p	27a-3p
F	296-5p	29a-3p	29b-3p	29c-3p	30b-5p	30c-5p	31-5p	320a	323a-5p	324-5p	326	328-3p
G	331-5p	335-5p	34a-5p	425-5p	451a	486-5p	7-5p	9-5p	9-3p	92a-3p	93-5p	96-5p
H	Ce	Ce	SN1	SN2	SN3	SN4	SN5	SN6	miRTC	miRTC	PPC	PPC

Figure 4.1 Expression of miRNAs in the serum of glioblastoma patients aged between 20 and 39 years. A) Average miRNA fold change in female glioblastoma patients aged between 20 and 39 years showed a number of miRNAs undetected in the sample set. The majority of the miRNAs dysregulated in this cohort were increased (red). B) Average fold change of miRNA in male glioblastoma patients aged between 20 and 39 years. The majority of miRNAs in this cohort were increased compared to gender and age matched controls. Heat map is representative of all three patient samples analysed showing all miRNAs scored A, B and C using online data analysis tool, line H of 96 well plate containing controls is not included in the heat maps. Ce: C. elegans primer assay, SN1-6: snoRNA/snRNA PCR controls, miRTC: Reverse transcription control, PPC: Positive PCR control.

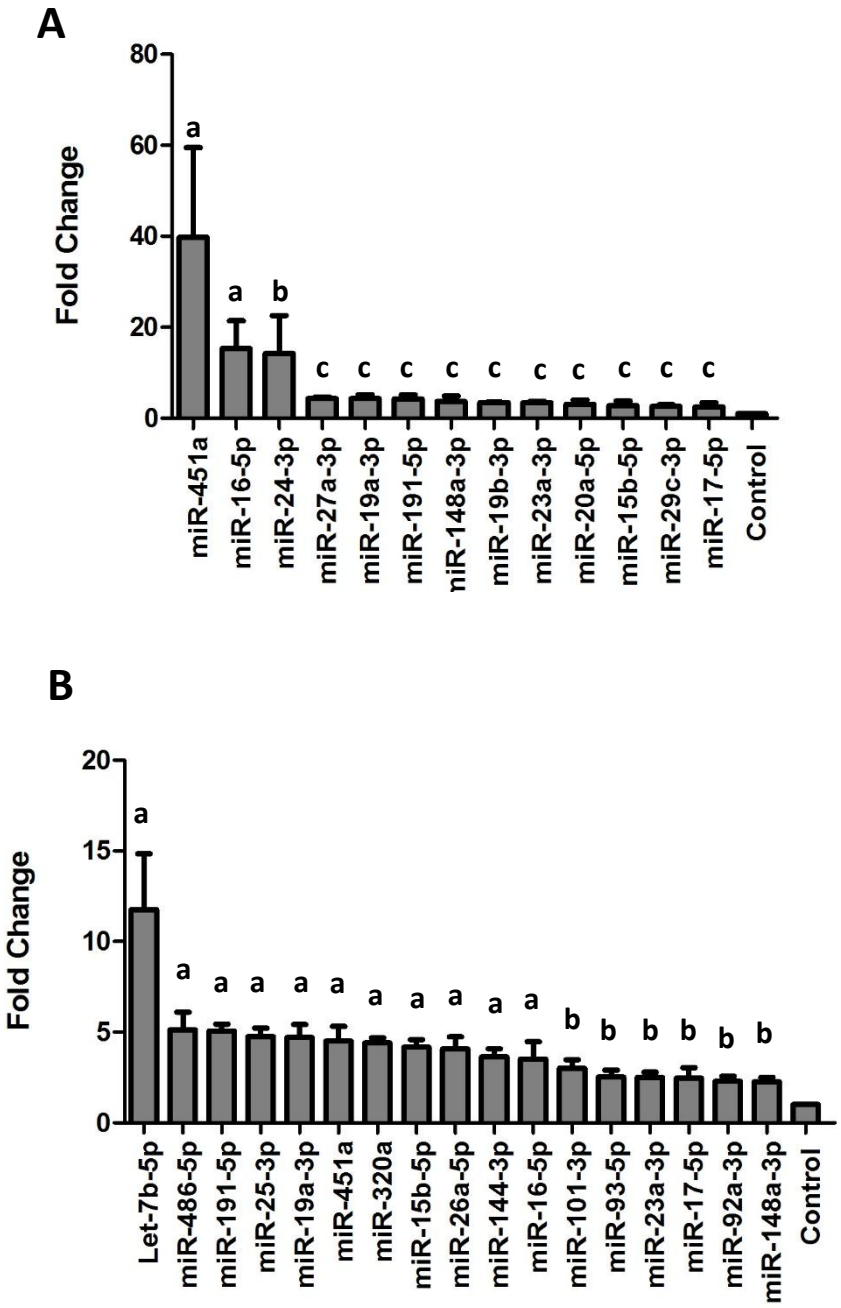


Figure 4.2 Expression of dysregulated miRNAs in the serum of glioblastoma patients aged between 20 and 39 years compared to age and sex matched controls. A) 13 miRNAs were increased in the serum of female glioblastoma patients between 20 and 39 years, with miR-451a showing the greatest increase in expression. B) 17 miRNAs were increased in the serum of male glioblastoma patients aged between 20 and 39 years with let-7b-5p showing the greatest increase in expression. No miRNAs were observed to be reduced in the male cohort. Data shown as mean of triplicate samples plus SD, representative of 3 samples per group, normalised to the cel-miR-39 spike in, with an average Ct scored either A or B using the online array tool. Significance between glioblastoma serum expression and control patient serum indicated by letters. a$p < 0.001$, b$p < 0.01$, c$p < 0.05$.

4.2.2 MiScript MicroRNA Expression in Serum Obtained from Glioblastoma Patients Aged between 40 and 59 Years.

Analysis of serum samples obtained from three female and three male glioblastoma patients aged between 40 and 59 years identified eight miRNAs expressed in all three samples from both the male and female 40-59 age groups (Table 4.2). Four of the eight miRNAs, let-7b-5p, 150-5p, 181b-5p and 92a-3p, displayed an increased up-regulation in male 40-59 serum samples compared to age matched female samples. The remaining four, 21-5p, 23a-3p, 25-3p and 191-5p, were expressed at higher levels in female samples compared to male age matched samples.

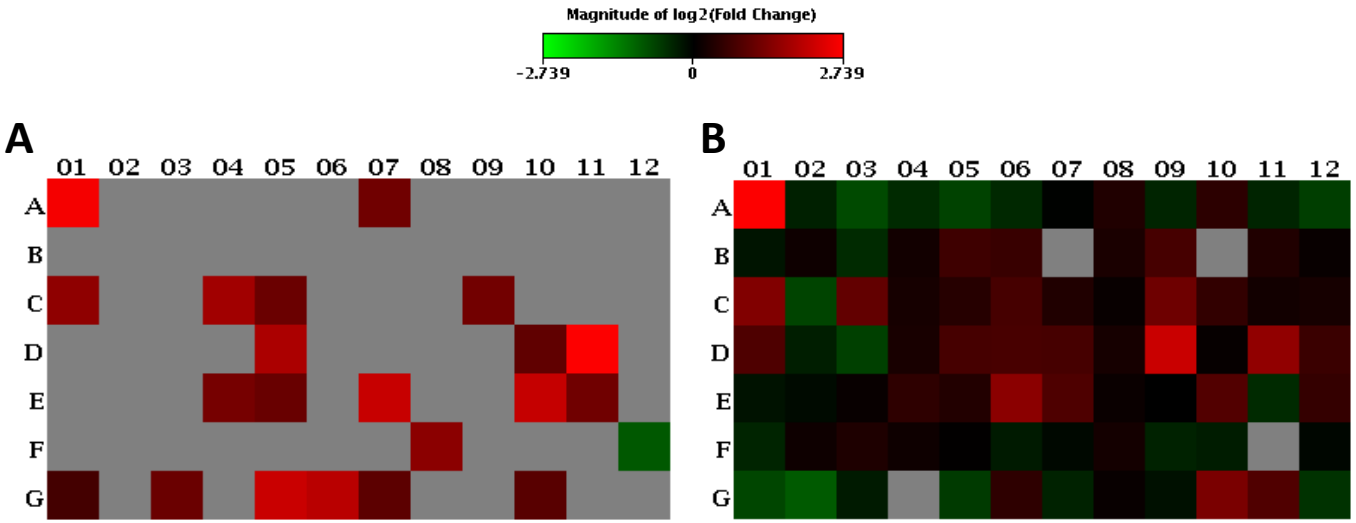
Most miRNAs with altered expression in all three female samples tested were up-regulated (Figure 4.3A). The majority of miRNAs analysed were undetected in the three samples tested from the female 40-59 group. A greater number of miRNAs were detected in the male 40-59 age group compared to the female group with four of the 84 miRNAs being undetected in all three samples (Figure 4.3B).

Serum obtained from female glioblastoma patients aged between 40 and 59 years showed an increase in expression of 20 miRNAs in all three samples compared to age and sex-matched control samples (Figure 4.4A). MiR-21-5p was the highest expressed miRNA in female 40-59 patients with a fold change of 8.59, was the second highest expressed miRNA was let-7b-5p with a fold change of 7.97. Three of the miRNAs showed a five-fold increase in expression, miR-451a, miR-23a-3p and miR-25-3p with a further two exhibiting a four-fold increase, miR-486-5p and 191-5p.

Out of the 84 miRNAs analysed, 18 were highly expressed in the three serum samples of male glioblastoma patients aged between 40 and 59 years (Figure 4.4B). Let-7b-5p had the highest expression with a fold change of 17.32 and miR-203a-3p being the second highest expressed miRNA with a fold change of 9.62. Mir-21-5p exhibited a 5.00 fold increase in expression with miR-222-5p and 150-5p showing a 4.00 fold increase in expression.

Table 4.2 MiRNAs dysregulated in the serum of both male and female glioblastoma patients aged between 40 and 59 years.

	Female		Male	
MiRNA	**Average Fold Change**	**SD**	**Average Fold Change**	**SD**
Let-7b-5p	7.97	2.43	17.32	6.21
MiR-150-5p	3.35	1.02	4.25	2.12
MiR-181b-5p	2.59	0.87	3.42	2.14
MiR-92a-3p	2.09	0.54	3.92	1.17
MiR-21-5p	8.59	0.72	5.11	1.60
MiR-23a-3p	5.44	1.42	2.38	1.02
MiR-25-3p	5.27	0.84	2.52	1.36
MiR-191-5p	4.27	0.26	2.20	1.81



	1	2	3	4	5	6	7	8	9	10	11	12
A	Let-7b-5p	101-3p	106b-5p	107	10b-5p	124-3p	125a-5p	125b-5p	127-5p	128-3p	129-5p	130a-3p
B	130b-3p	132-3p	133a-3p	133b	137	138-5p	141-3p	144-3p	146a-5p	146b-5p	148a-3p	149-5p
C	150-5p	153-3p	15a-5p	15b-5p	16-5p	17-5p	17-3p	181a-5p	181b-5p	182-5p	183-5p	184
D	185-5p	187-3p	18a-5p	190a-5p	191-5p	19a-3p	19b-3p	200a-3p	203a-3p	20a-5p	21-5p	210-3p
E	216a-5p	217	218-5p	221-3p	222-3p	222-5p	23a-3p	23b-3p	24-3p	25-3p	26a-5p	27a-3p
F	296-5p	29a-3p	29b-3p	29c-3p	30b-5p	30c-5p	31-5p	320a	323a-5p	324-5p	326	328-3p
G	331-5p	335-5p	34a-5p	425-5p	451a	486-5p	7-5p	9-5p	9-3p	92a-3p	93-5p	96-5p
H	Ce	Ce	SN1	SN2	SN3	SN4	SN5	SN6	miRTC	miRTC	PPC	PPC

Figure 4.3 Expression of miRNAs in serum samples from glioblastoma patients aged between 40 and 59 years. A) Average miRNA fold change in the serum of female patients. The majority of dysregulated miRNAs were found to be up-regulated (red) in this patient cohort however most miRNAs within the array were not detected in this sample set. B) Average miRNA fold change in the serum of male patients. Four miRNAs were not detected in this patient cohort, many of the miRNAs that were detected in this cohort showed a similar expression to matched controls. Heat map is representative of all three patient samples analysed showing all miRNAs scored A, B and C using online data analysis tool, line H of 96 well plate containing controls is not included in the heat maps. Ce: C. elegans primer assay, SN1-6: snoRNA/snRNA PCR controls, miRTC: Reverse transcription control, PPC: Positive PCR control.

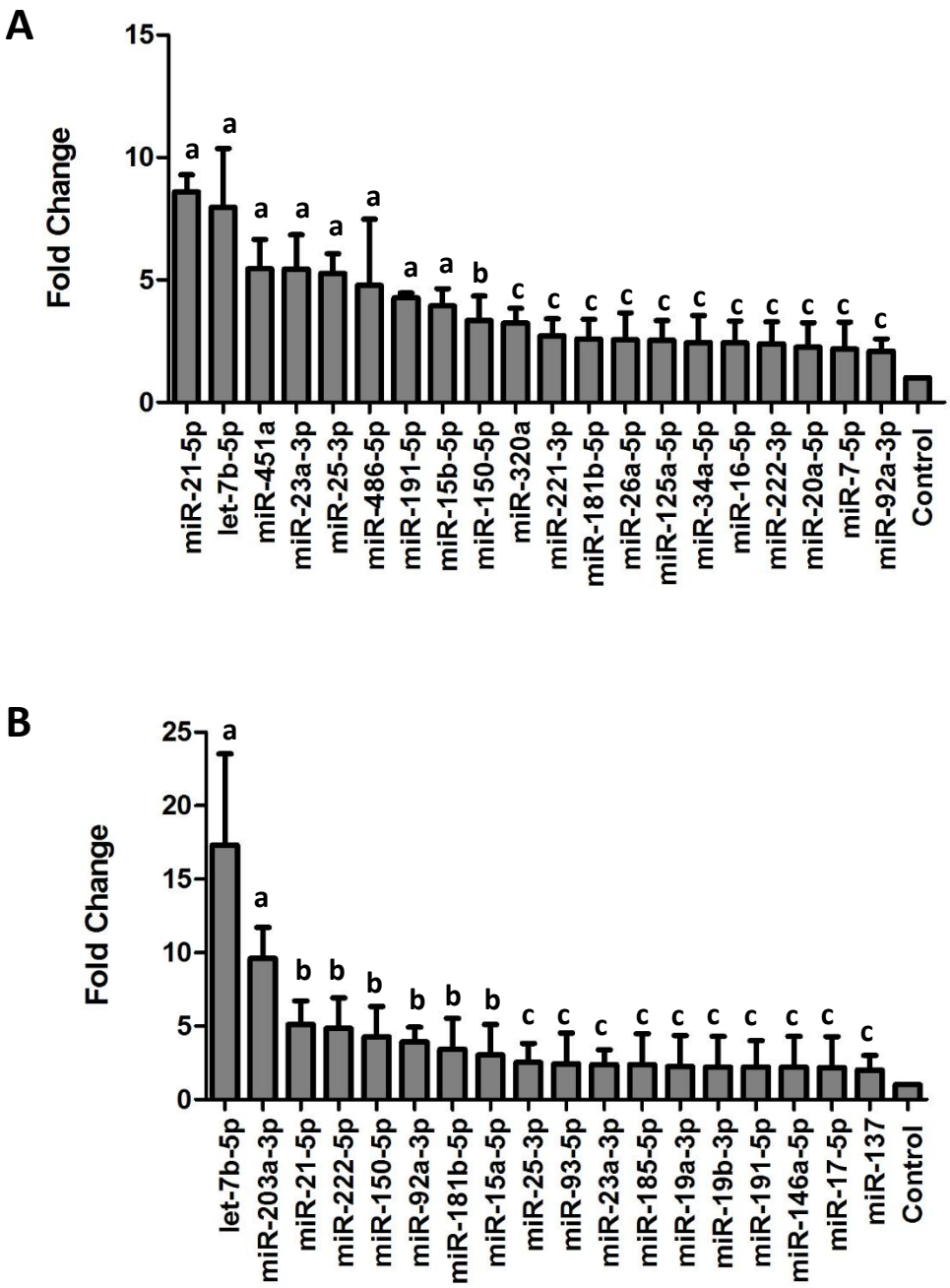


Figure 4.4 Expression of dysregulated miRNAs in glioblastoma patients aged between 40 and 59 years. A) 20 miRNAs were up-regulated in the serum of female glioblastoma patients in the 40-59 age group with miR-21-5p exhibiting the highest fold change. B) 16 miRNAs were up-regulated in the serum of male glioblastoma patients between the ages of 40 and 59 years. Let-7b-5p exhibited the highest up-regulation with a fold change of 17.32. Data shown as mean of triplicate samples plus SD, representative of 3 samples per group, of miRNAs scored either A or B using the online array tool, normalised to the cel-miR-39 spike in. Significance between glioblastoma serum expression and control patient serum indicated by letters. a$p < 0.001$, b$p < 0.01$, c$p < 0.05$.

4.2.3 MiScript MicroRNA Expression in Serum Obtained from Glioblastoma Patients Aged Over 60 Years.

Analysis of serum obtained from glioblastoma patients aged over 60 years identified a total of seven miRNAs dysregulated in the six serum samples of both male and female glioblastoma patients over the age of 60 years (Table 4.3). Comparison of the seven miRNAs identified five miRNAs which exhibited a higher expression in the male 60+ glioblastoma group compared to age matched females except miR-9-3p and miR-29c-3p which were expressed at a higher level in the female group.

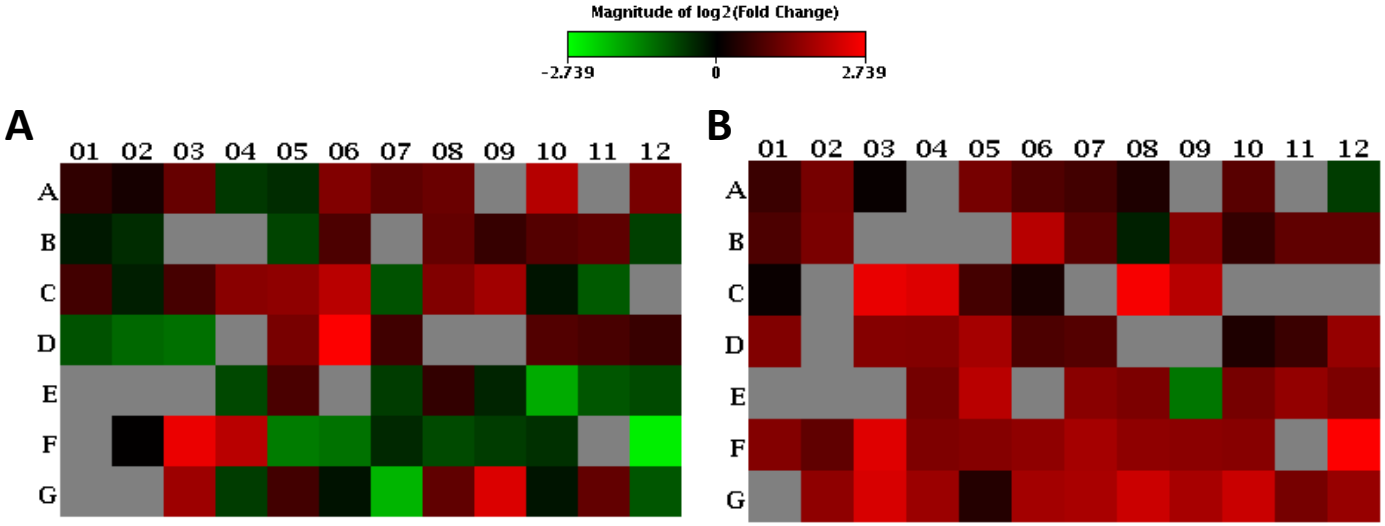
An overall trend of reduced expression in miRNAs compared to age and sex-matched controls was observed in all three samples obtained from female glioblastoma patients when all miRNAs were investigated regardless of Ct score (Figure 4.5A). Analysis of the dysregulated miRNAs scored A or B only showed 10 miRNAs with an increase in expression and no miRNAs with a lower expression compared to age and sex-matched controls (Figure 4.6). Upon analysis of serum miRNA expression in male glioblastoma patients aged over 60 years increased expression of 42 miRNAs in all three serum samples compared to age and sex-matched controls was observed (Figure 4.5B). Overall the male group displayed a general trend of increased miRNA expression compared to age and sex-matched controls.

In the female group, miR-19a-3p and miR-29b-3p showed the greatest increase in expression compared to matched controls with a fold change of 4.99 and 4.45 respectively (Figure 4.6). Four miRNAs showed a 3.00 fold increase in the female group, miR-9-3p, miR-17-5p, miR-29c-3p and miR-128-3p.

Analysis of the male group identified miR-328-3p and 181a-5p as having the greatest increase in expression in comparison to matched controls with a fold change of 6.22 and 5.9, respectively (Figure 4.7). Two miRNAs exhibited a five-fold increase, miR-181a-5p and miR-15a-5p and five miRNAs showed a four-fold increase, miR-29b-3p, 15b-5p, 34a-5p, miR-92a-3p and miR-9-5p. Nine miRNAs showed approximately a three fold increase in expression and 25 miRNAs showed at least a two fold increase in expression.

Table 4.3 MiRNAs dysregulated in the serum of both male and female glioblastoma patients aged over 60 years compared to age and sex matched controls.

	Female		Male	
MiRNA	**Average Fold Change**	**SD**	**Average Fold Change**	**SD**
miR-9-3p	3.98	0.51	3.33	0.49
miR-15b-5p	2.38	0.65	4.91	0.27
miR-29b-3p	4.45	0.87	4.99	0.58
miR-29c-3p	3.22	0.74	2.48	0.27
miR-34a-5p	2.72	0.81	4.70	0.43
miR-181a-5p	2.28	1.30	5.90	0.68
miR-181b-5p	2.78	0.73	3.72	0.35



	1	2	3	4	5	6	7	8	9	10	11	12
A	Let-7b-5p	101-3p	106b-5p	107	10b-5p	124-3p	125a-5p	125b-5p	127-5p	128-3p	129-5p	130a-3p
B	130b-3p	132-3p	133a-3p	133b	137	138-5p	141-3p	144-3p	146a-5p	146b-5p	148a-3p	149-5p
C	150-5p	153-3p	15a-5p	15b-5p	16-5p	17-5p	17-3p	181a-5p	181b-5p	182-5p	183-5p	184
D	185-5p	187-3p	18a-5p	190a-5p	191-5p	19a-3p	19b-3p	200a-3p	203a-3p	20a-5p	21-5p	210-3p
E	216a-5p	217	218-5p	221-3p	222-3p	222-5p	23a-3p	23b-3p	24-3p	25-3p	26a-5p	27a-3p
F	296-5p	29a-3p	29b-3p	29c-3p	30b-5p	30c-5p	31-5p	320a	323a-5p	324-5p	326	328-3p
G	331-5p	335-5p	34a-5p	425-5p	451a	486-5p	7-5p	9-5p	9-3p	92a-3p	93-5p	96-5p
H	Ce	Ce	SN1	SN2	SN3	SN4	SN5	SN6	miRTC	miRTC	PPC	PPC

Figure 4.5 Expression of miRNA in serum obtained from glioblastoma patients over the age of 60 years. A) Average miRNA expression in the serum of female patients showed both a down-regulation (green) and up-regulation (red) of miRNAs compared to gender and age-matched controls. 17 miRNAs were not detected in this patient cohort. B) Average miRNA fold change in the serum of male patients. The majority of dysregulated miRNAs were found to be up-regulated in this patient cohort and 20 miRNAs were not detected in this sample set. Heat map is representative of all three patient samples analysed showing all miRNAs scored A, B and C using online data analysis tool, line H of 96 well plate containing controls is not included in the figure.

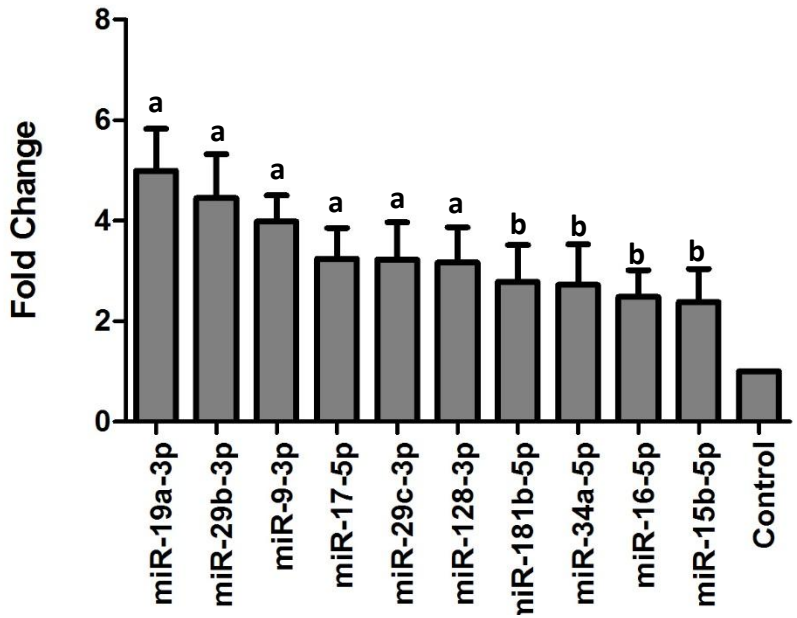


Figure 4.6 Dysregulated miRNAs in the serum of female glioblastoma patients aged over 60 years. 10 miRNAs were increased in the serum of female 60+ glioblastoma patients with miR-19a-3p exhibiting the highest fold-change. Data shown as mean of triplicate samples plus SD, representative of 3 samples per group of miRNAs scored either A or B using the online array tool, normalised to the cel-miR-39 spike in. Significance between glioblastoma serum expression and control patient serum indicated by letters. a$p < 0.001$, b$p < 0.01$.

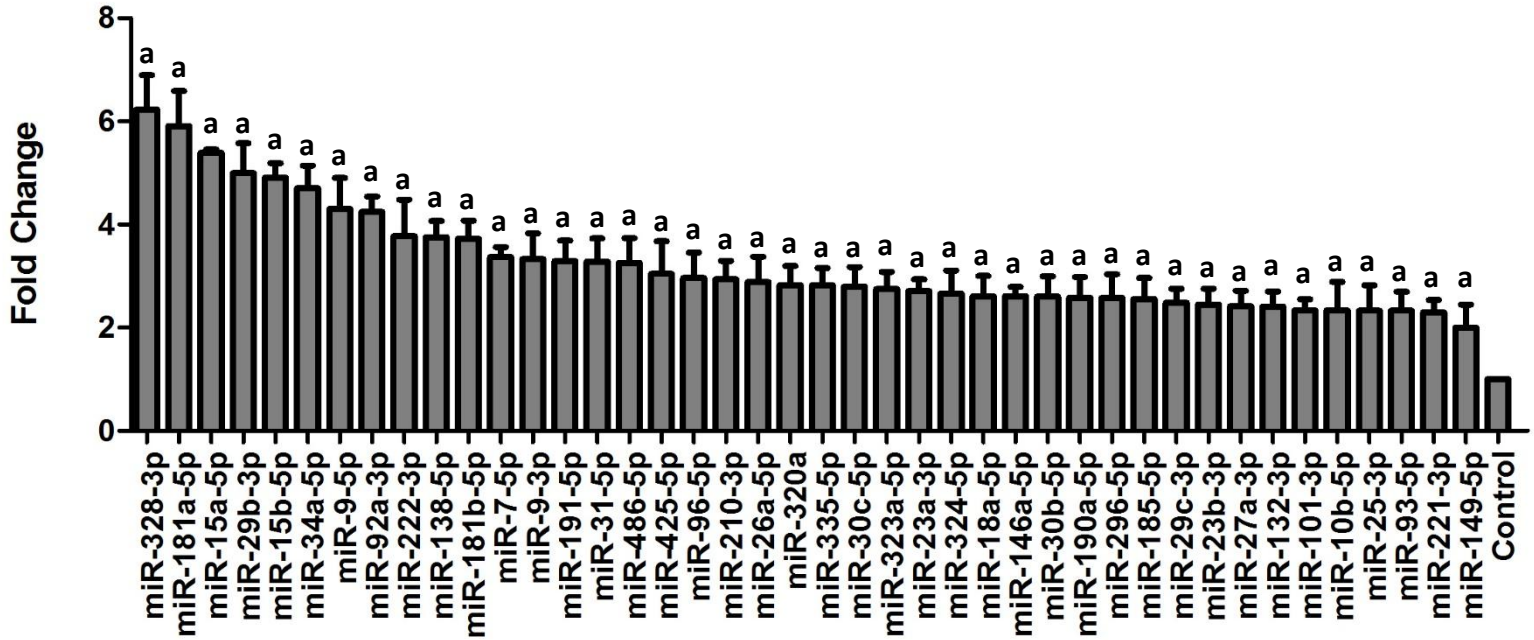


Figure 4.7 Dysregulated miRNAs in the serum of male glioblastoma patients aged over 60 years. 42 miRNAs were increased in the serum of male glioblastoma patients aged over 60 years, with miR-328-3p showing the greatest increase in expression. Data shown as mean of triplicate samples plus SD, representative of 3 samples per group, of miRNAs scored either A or B using the online array tool, normalised to the cel-miR-39 spike in. Significance between glioblastoma serum expression and control patient serum indicated by letters. a$p < 0.001$.

4.2.4 Selection of Serum MicroRNA Biomarkers

Following identification of miRNAs with altered expression in the sera of glioblastoma patients grouped by age and gender, a panel was selected for further analysis in a new patient cohort of 18 GBM patients and 18 control patients. Due to the small sample size used in the initial study, miRNAs with altered expression in more than three patient groups or both gender cohorts of the same age group were selected for this study. Following these criteria, 32 miRNAs were selected for analysis in a new patient cohort (Table 4.4).

Analysis of the 32 miRNAs selected in the new patient cohort using student's t-test with Levene's F test identified 18 miRNAs that were undetected in any of the patient serum samples grouped by age and gender (Table 4.5). Analysis of the 32 miRNAs identified nine miRNAs with no significant change ($p > 0.05$) in expression in glioblastoma patient serum compared to age and sex matched controls. Comparison of the non-significant miRNAs in glioblastoma patient serum compared to control patient serum is shown in Figure 4.8. Data were further analysed by gender and age groups which also showed no significant difference ($p > 0.05$) in expression (Appendix 3). Five miRNAs were identified to have a significant change in expression in the serum of glioblastoma patients, miR-20a-5p, miR-30c-5p, miR-34a-5p, miR-92a-3p and miR-150-5p. MiR-34a expression was not significantly different ($p > 0.05$) in the serum of glioblastoma patients compared to control patients (Figure 4.9A). Analysis of patients grouped by gender showed no significant difference ($p > 0.05$) in miR-34a expression in both male and female glioblastoma patient cohorts (Figure 4.9 B and C). When grouped by age miR-34a was not detected in the serum of glioblastoma patients or control patients aged between 20 and 39 years. MiR-34a expression was not significantly different ($p > 0.05$) between glioblastoma patients aged between 40 and 59 years compared to age matched controls (Figure 4.9D). Expression of miR-34a in the serum of glioblastoma patients aged over 60 years was found to be significantly up-regulated ($p < 0.05$) compared to age matched controls (Figure 4.9E). Comparison of miR-92a expression in glioblastoma patient serum compared to control

patient serum showed no significant difference ($p > 0.05$) between the two cohorts (Figure 4.10A).

Table 4.4 MiRNAs selected for analysis as biomarkers and average fold change of all patient groups. Red – Increased expression, Green – Decreased expression, Black – No change in expression compared to control samples.

MiRNA	Female 60+	Male 60+	Female 40-59	Male 40-59	Female 20-39	Male 20-39
let-7-5p	1.35	1.52	7.97	17.32	0.54	11.76
7-5p	0.31	3.36	2.18	0.68	-	0.92
9-3p	3.98	3.33	-	0.84	-	-
15b-5p	2.38	4.91	3.94	3.02	2.76	4.17
16-5p	2.48	1.63	2.43	1.53	15.37	3.53
17-5p	3.24	1.21	-	2.17	2.47	2.46
18a-5p	0.49	2.61	-	0.48	-	1.59
19a-3p	4.99	1.71	-	2.25	4.38	4.71
19b-3p	1.50	1.81	-	2.21	3.48	2.18
20a-5p	1.67	1.24	2.25	1.05	3.12	2.05
21-5p	1.57	1.06	8.59	4.25	1.17	1.76
23a-3p	0.68	2.70	5.44	2.38	3.43	2.50
25-3p	0.33	2.32	5.27	2.52	0.80	4.75
26a-5p	0.58	2.88	2.56	0.62	1.93	4.07
29b-3p	4.45	4.99	-	1.38	-	1.21
29c-3p	3.22	2.48	-	1.15	2.61	1.68
30b-5p	0.45	2.60	-	1.01	1.71	0.93
30c-5p	0.48	2.79	-	0.74	1.81	1.25
34a-5p	2.72	4.70	2.44	0.75	-	-
92a-3p	0.88	4.24	2.09	3.92	0.50	2.3
93-5p	1.85	2.32	-	2.43	2.00	2.52
101-3p	1.14	2.33	-	0.70	2.01	3.00
148a-3p	1.81	1.99	-	1.44	3.68	2.25
150-5p	1.50	1.06	3.35	4.25	1.12	1.74
181a-5p	2.28	5.90	-	1.09	-	0.69
181b-5p	2.78	3.72	2.59	3.42	1.15	0.79
185-5p	0.59	2.55	-	2.37	1.36	2.11
191-5p	2.12	3.28	4.27	2.20	4.26	5.07
320a	0.62	2.82	3.24	1.26	1.96	4.40
328-3p	0.22	6.22	0.47	0.92	1.71	1.11
451a	1.51	1.29	5.46	0.51	39.76	4.51
486-5p	0.88	3.25	4.78	1.66	0.07	5.13

Table 4.5 MiRNAs undetected in glioblastoma patient serum samples in new patient cohort analysed using miScript brain cancer panel.

Undetected miRNAs	
miR-7-5p	**miR-26a-5p**
let-7b-5p	**miR-29b-3p**
miR-9-3p	**miR-29c-3p**
miR-15b-5p	**miR-30b-5p**
miR-16-5p	**miR-93-5p**
miR-18a-5p	**miR-148a-3p**
miR-19a-3p	**miR-181a-5p**
miR-19b-3p	**miR-185-5p**
miR-21-5p	**miR-191-5p**

Analysis of miR-92a by gender showed that miR-92a was significantly up-regulated in the sera of male glioblastoma patients ($p < 0.05$) but not in female glioblastoma patients ($p > 0.05$, Figures 4.10B and C). Analysis of miR-92a by age groups showed no significant difference in expression ($p > 0.05$) compared to age matched controls for all three patient cohorts (Figure 4.10D-F).

Analysis of miR-20a expression displayed a significant up-regulation ($p < 0.05$) in the serum of glioblastoma patients compared to control patients (Figure 4.11A). When grouped by gender miR-20a was significantly increased in the serum of both male ($p < 0.05$) and female ($p < 0.05$) glioblastoma patients compared to sex matched controls (Figure 4.11 B and C). Analysis of miR-20a by age showed a significant increase of miR-20a in the 20-39 ($p < 0.001$) and 40-59 ($p < 0.05$) age groups (Figure 4.11 D and E). Expression of miR-20a in the serum of glioblastoma patients over the age of 60 years was not significantly different ($p > 0.05$) to age matched controls (Figure 4.11F). Further analysis of miR-20a expression in glioblastoma patient serum identified a subgroup of nine patients who did not exhibit an increase in miR-20a and nine patients who exhibited an up-regulation of miR-20a ($p > 0.05$, Figure 4.12A). Expression of miR-20a was significantly increased in glioblastoma patients between the ages of 20 and 39 years compared to those aged between 40 and 59 years ($p < 0.01$) and those aged over 60 years ($p < 0.001$, Figure 4.12B). No significant difference ($p > 0.05$) in expression of miR-20a was observed between the 40-59 age group and the 60+ age group.

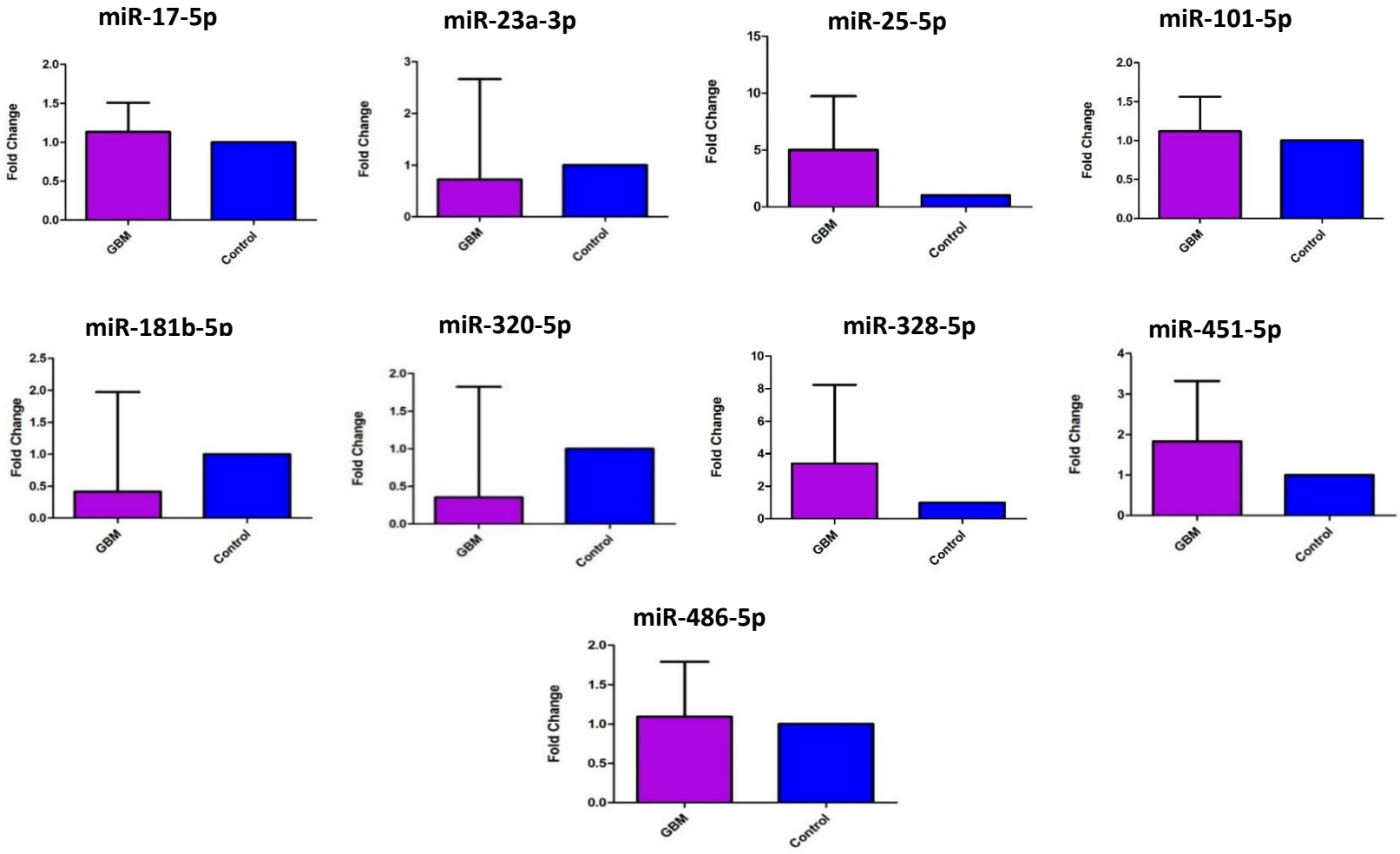


Figure 4.8 Selected serum miRNAs with no significant change in expression. Nine miRNAs exhibited no significant change in expression in GBM patient serum compared to matched controls. Data shown as mean of triplicate samples plus SD analysed by unpaired student's t-test, miR-486-5p analysed by Mann Whitney U test, $p > 0.05$.

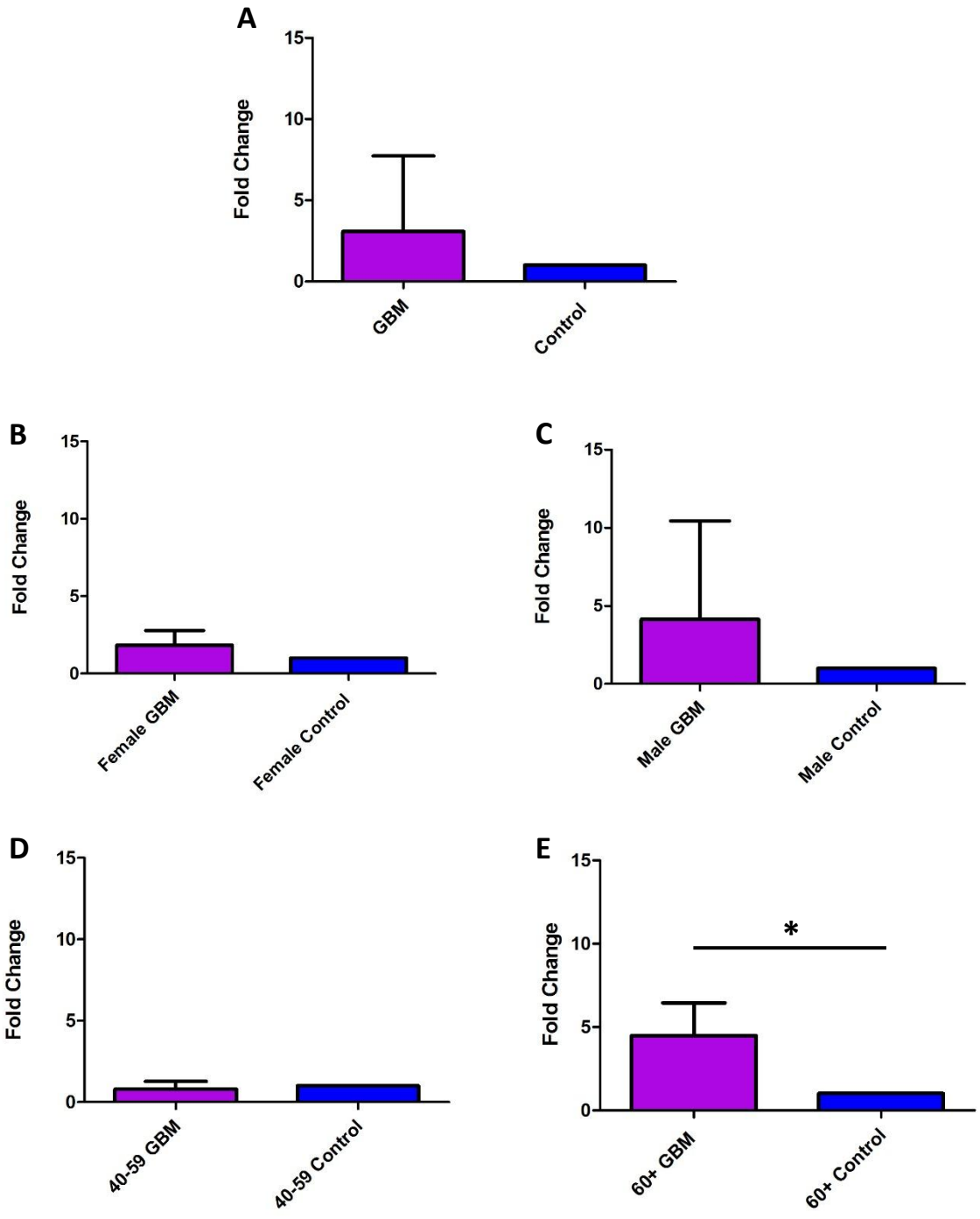


Figure 4.9 Expression of miR-34a in glioblastoma patients aged over 60 years. Expression of miR-34a was not significantly different between GBM and control patients (A) or when grouped by gender (B and C).Expression of miR-34a was not significantly different between GBM patients aged between 40 and 59 years and age matched controls (D). MiR-34a was significantly up-regulated in the serum of glioblastoma patients aged over 60 years compared to age matched controls (E). Data shown as mean plus SD of replicate samples (A - n = 18, B and C - n = 9, D and E - n = 6 per group), data analysed by Mann Whitney U test. Significance indicated between groups by solid black lines, * $p < 0.05$.

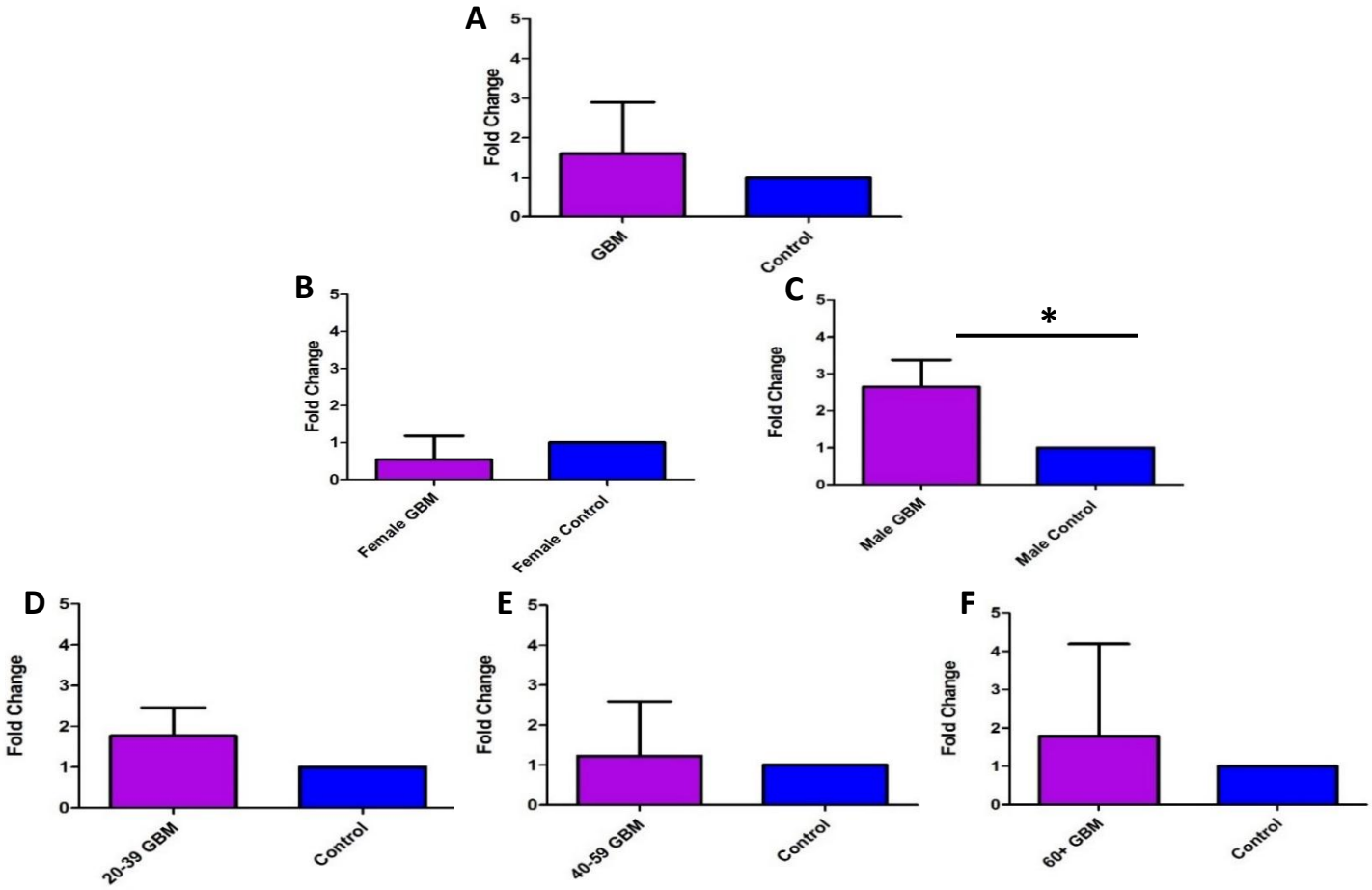


Figure 4.10 Expression of miR-92a in serum. Expression of miR-92a was not significantly altered in the serum of GBM patients compared to controls (A) or female GBM patients (B). MiR-92a was significantly increased in the serum of male GBM patients compared to sex matched controls (C). Expression of miR-92a was not significantly different when patients were grouped by age (D-F). Data shown as mean of replicates (A - n = 18, B and C - n = 9, D-F - n = 6 per group) plus SD, analysed by unpaired student's t-test. Significance indicated between groups by solid black lines, * $p < 0.05$.

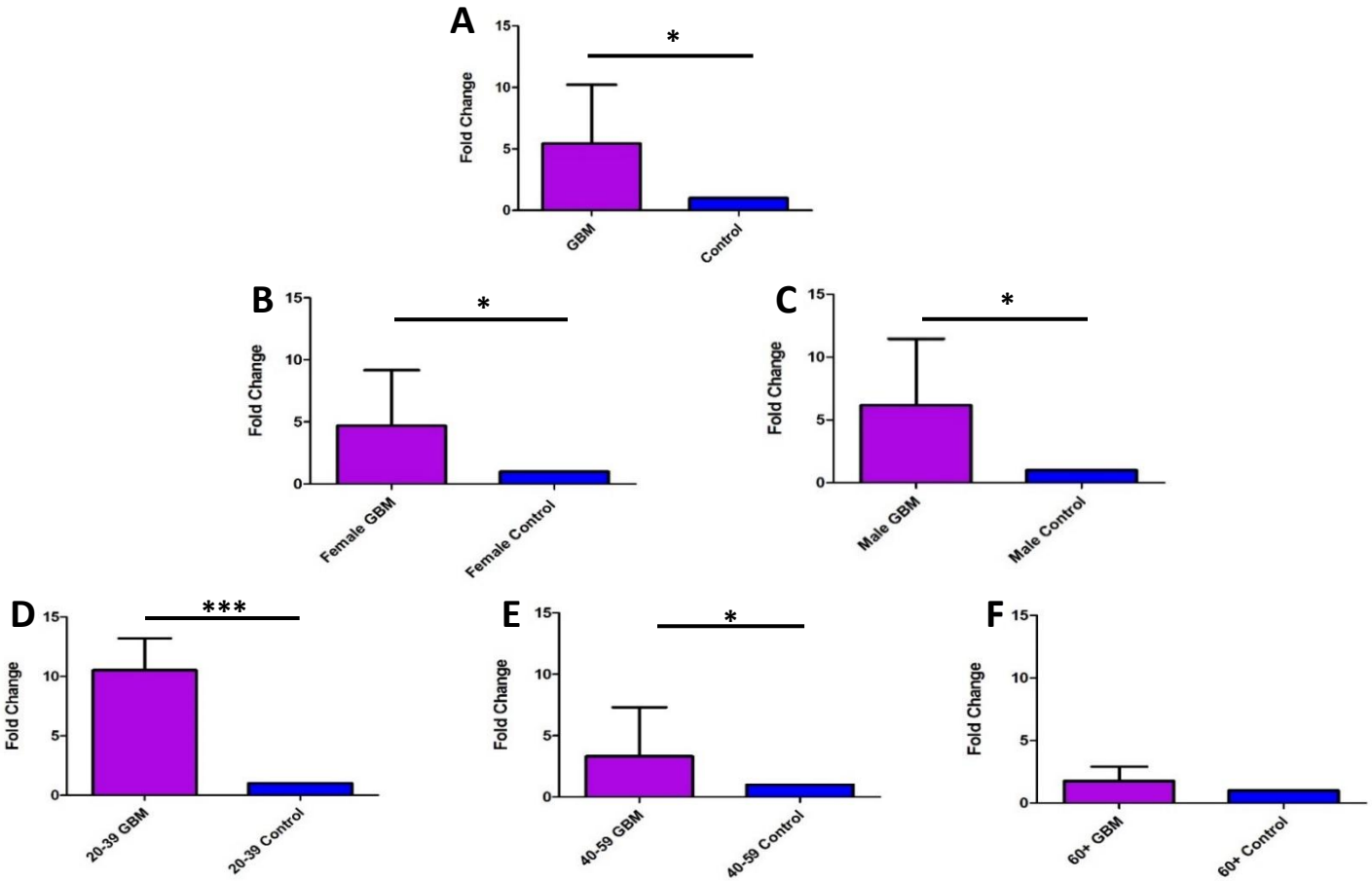


Figure 4.11 Expression of miR-20a in serum. MiR-20a was significantly increased in glioblastoma patient serum compared to controls (A). MiR-20a was significantly up-regulated in both male and female patients (B and C). When grouped by age miR-20a was significantly increased in GBM patients in the 20-39 (D) and 40-59 (E) age groups but not the 60+ age group (F). Data shown as mean of replicates (A - n = 18, B and C - n = 9, D-F - n = 6 per group) plus SD, A - analysed by unpaired student's t-test B-F analysed by Mann Whitney U test. Significance indicated between groups by solid black lines, * $p < 0.05$, *** $p < 0.001$.

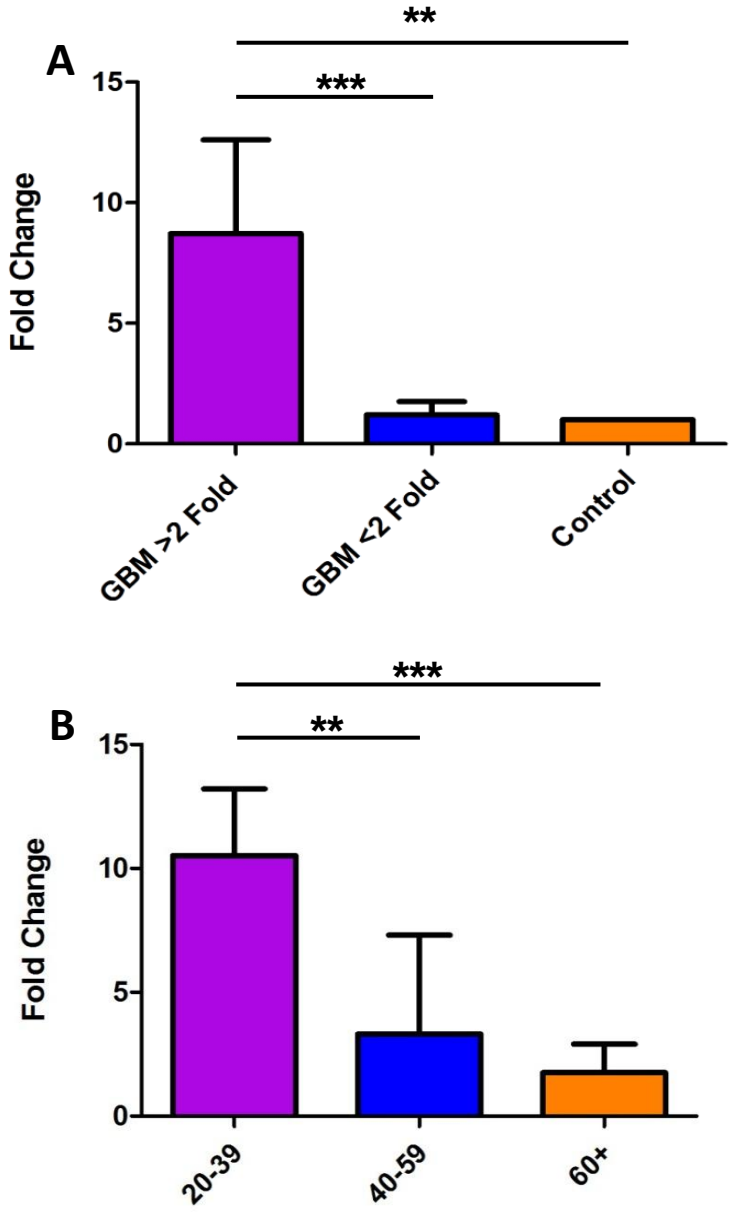


Figure 4.12 Expression of miR-20a in the serum of glioblastoma patient subgroups. A) Two groups of GBM patients were observed with differing miR-20a expression, those with an increase in expression (> 2 fold change, n = 9) and those with no change (< 2 fold change, n = 9). B) Expression of miR-20a was inversely correlated with age with GBM patients aged between 20 and 39 years exhibiting the highest expression. Data shown as mean of replicate samples (A- n = 18 GBM, 18 control, B - n=6 per age group) plus SD, analysed by one-way ANOVA with Tukey *post-hoc* test. Significance between groups indicated by a solid black line, $**p < 0.01$, $***p < 0.001$.

The subgroup of glioblastoma patients with an increase of miR-20a comprised all six patients aged between 20 and 39 years, one patient aged between 40 and 59 years and two patients aged over 60 years. The subgroup of glioblastoma patients with no change in miR-20a expression comprised of five patients aged between 40 and 59 years and four patients aged over 60 years.

Analysis of miR-30c exhibited a significant down-regulation ($p < 0.01$) in the serum of both male and female glioblastoma patients aged between 20 and 39 years. MiR-30c was not detected in either the 40-59 or 60+ age groups (Figure 4.13). MiR-150 was observed to be significantly down-regulated ($p < 0.05$) in the serum of both male and female glioblastoma patients aged between 20 and 39 years. MiR-30c was not detected in the 40-59 or 60+ glioblastoma patient age groups (Figure 4.14).

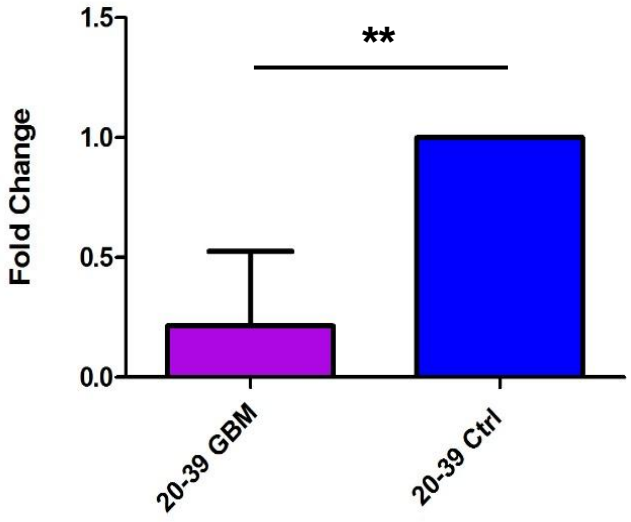


Figure 4.13 Expression of miR-30c in the serum of glioblastoma patients aged between 20 and 39 years. MiR-30c was down-regulated in the serum of GBM patients aged between 20 and 39 years. Data shown as mean of replicates (n=6 per group) plus SD, analysed unpaired student's t-test. Significance between groups indicated by a solid black line, $**p < 0.01$.

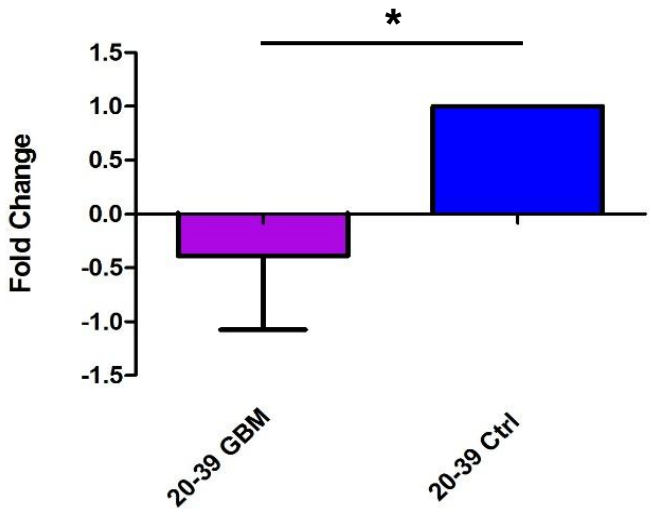


Figure 4.14 Expression of miR-150 in the serum of glioblastoma patients aged between 20 and 39 years. Expression of miR-150 was significantly down-regulated in the serum of GBM patients aged between 20 and 39 years compared to matched controls. Data shown as mean of replicate samples (n=6 per group) plus SD, analysed by unpaired student's t-test. Significance between groups indicated by a solid black line, $*p < 0.05$.

4.2.5 Validation of MicroRNA Biomarkers by Power Analysis

Following the identification of a panel of potential miRNA biomarkers, the five miRNAs were validated in a further study using power analysis to determine the appropriate sample size to ensure confidence in any significant changes observed in expression. Power analysis was performed using GraphPad prism Statmate and standard deviation of ΔCt values for each miRNA were used to determine sample size for at least 80 % power to detect a difference between means of 1 Ct value (Table 4.6).

Table 4.6 Sample number required in each experimental group required for a minimum of 80 % power.

MiRNA	Standard Deviation	Sample Size Required	Statistical Power
miR-34a-5p	0.65	8	90 %
miR-92a-3p	1.4	35	80 %
miR-20a-5p	0.85	14	90 %
miR-30c-5p	0.21	3	80 %
miR-150-5p	0.68	9	80 %

The power analysis determined that eight samples were required in each group for miR-34a, 14 for miR-20a-5p, 35 for miR-92a-3p, three for miR-30c-5p and nine for miR-150-5p. Data from the validation study showed that miR-34a was increased in the serum of glioblastoma patients aged over 60 years compared to age matched controls ($p < 0.001$, Figure 4.15). MiR-34a was undetected in the 20-39 patient group, and expression was not significantly altered in the 40-59 age group ($p > 0.05$), compared to age matched controls. MiR-92a was significantly increased ($p < 0.001$) in the validation cohort of male glioblastoma patient serum (Figure 4.16).

MiR-30c was found to be significantly reduced ($p < 0.01$) in the serum of glioblastoma patients aged between 20 and 39 years (Figure 4.17). Analysis of miR-20a expression showed an up-regulation ($p < 0.01$) in a subset of ten patients (Figure 4.18A) and no up-regulation in five patients however this was not significantly different compared to control.

Expression was highest in glioblastoma patients aged between 20 and 39 years, and lowest in patients aged over 60 years (Figure 4.18B). The patient subgroup with an increase of miR-20a comprised of all five glioblastoma patients aged between 20 and 39 years, three patients aged between 40 and 59 years and two patients aged over 60 years. MiR-150 expression was not found to be significantly different ($p > 0.01$) in the serum of glioblastoma patients aged between 20 and 39 years in the validation cohort (Figure 4.19).

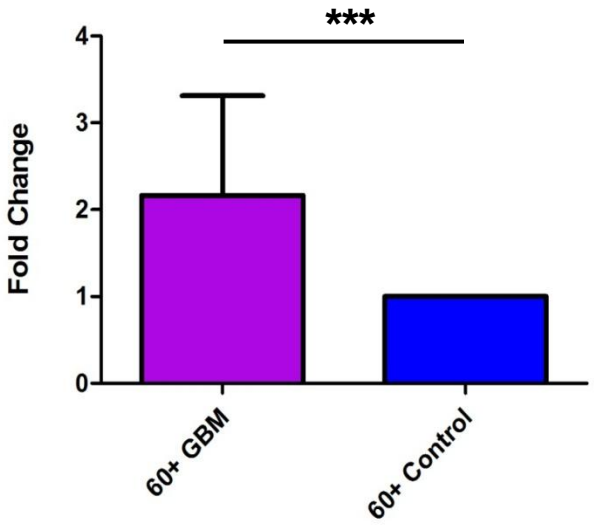


Figure 4.15 Expression of miR-34a in validation cohort. MiR-34a was significantly higher in a validation cohort of serum from GBM patients over the age of 60 years compared to age matched controls. Data shown as mean of replicate samples (n = 8 per group) plus SD, analysed by unpaired student's t-test. Significance between groups indicated by a solid black line, ***$p < 0.001$.

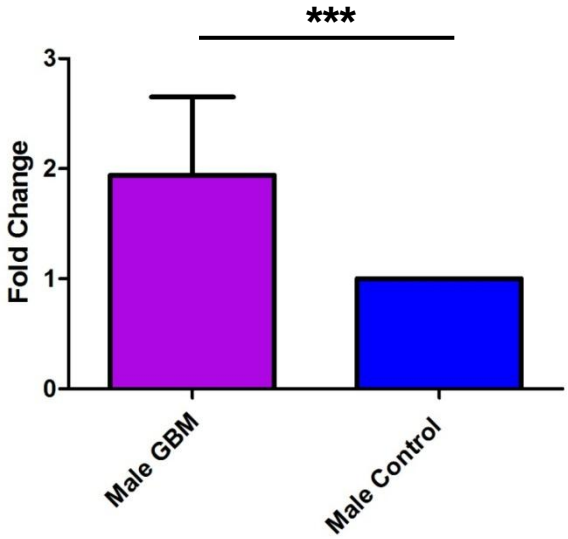


Figure 4.16 Expression of miR-92a in validation cohort. MiR-92a was shown to be significantly higher in a validation cohort of serum samples from male GBM patients compared to matched controls. Data shown as mean of replicate samples (n=35 per group) plus SD, analysed by unpaired student's t-test. Significance between groups indicated by a solid black line ***$p < 0.001$.

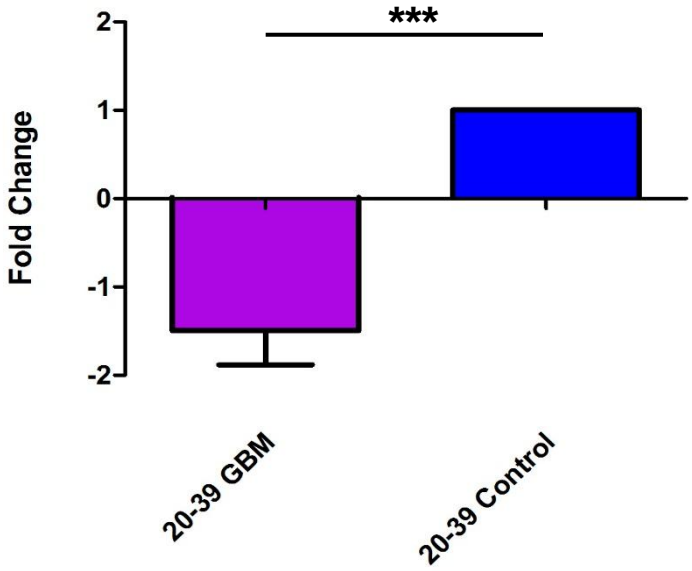


Figure 4.17 Validation of miR-30c expression in the serum of glioblastoma patients aged between 20 and 39 years. Expression of miR-30c in the validation cohort exhibited a significant reduction. Data shown as mean of replicate samples (n = 3 per group) plus SD, analysed by unpaired student's t-test. Significance between groups indicated by a solid black line, ***$p < 0.001$.

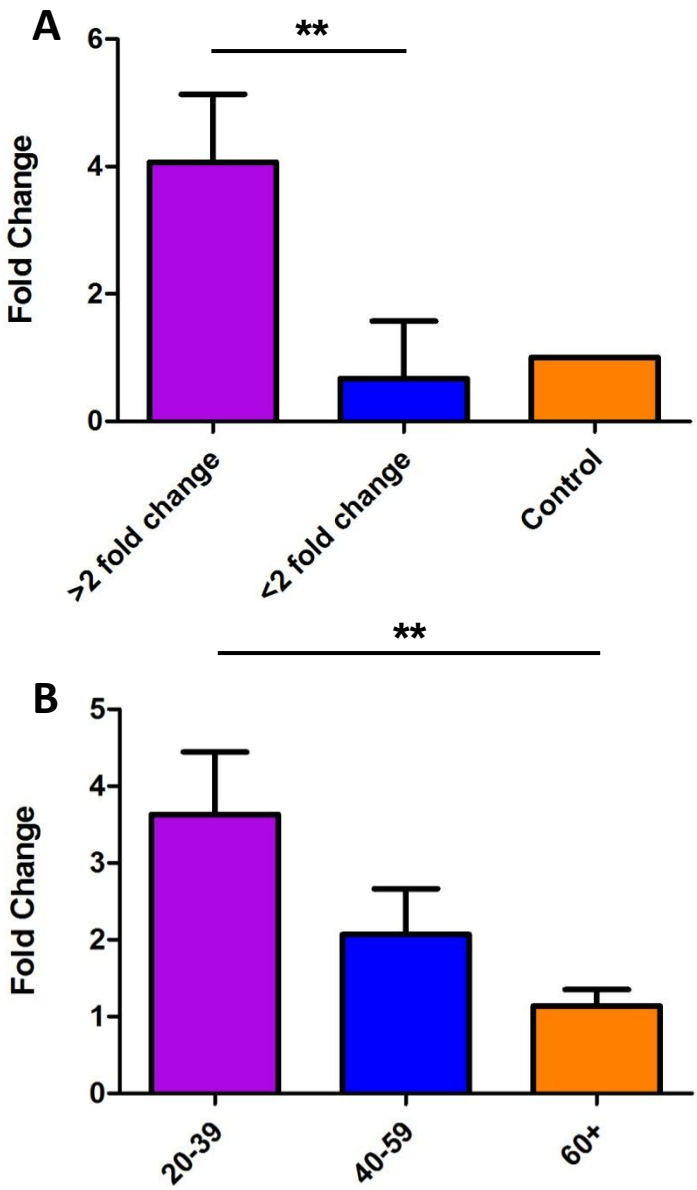


Figure 4.18 Expression of miR-20a in validation cohort. A) Serum miR-20a was observed to be significantly increased in a subset of ten GBM patients in the validation cohort however this was not significantly different to control patients. Five patients exhibited a less than 2-fold change in expression B) Expression of miR-20a in the validation cohort was found to be inversely correlated with age. Data shown as mean of replicate samples (A - n= 15 gbm and 15 control, B - n= 5 per age group) plus SD, analysed by Shapiro-Wilk normality test and one way ANOVA with Tukey *post-hoc* test. Significance indicated by a solid black line, $^{**}p < 0.01$.

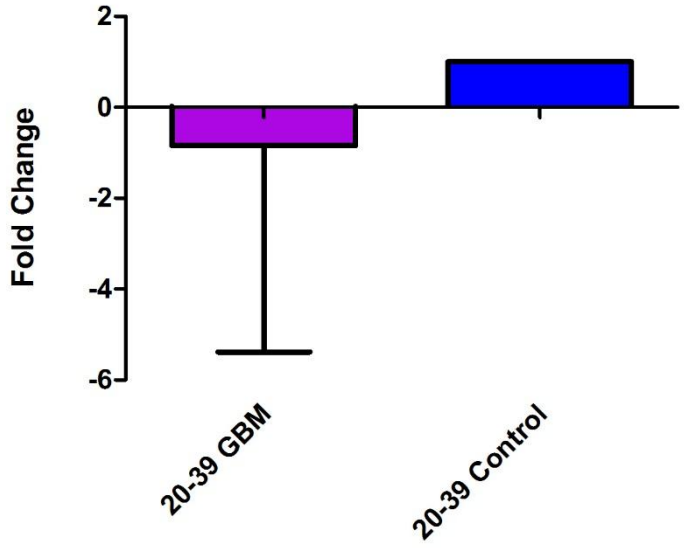


Figure 4.19 Validation of miR-150 expression in the serum of glioblastoma patients aged between 20 and 39 years. Expression of miR-150 in the validation cohort showed no significant difference between 20-39 GBM patient serum and matched controls. Data shown as mean of replicated (n= 9 per group) plus SD, analysed by unpaired student's t-test, $p > 0.05$.

4.2.6 Reanalysis of Unselected MiRNAs

Analysis and validation of the 32 miRNAs identified in Section 4.2.4 identified four miRNAs with a significant change in expression in glioblastoma patient serum. The 27 remaining miRNAs which were not significantly different in Section 4.2.4 were used to select a small panel for reanalysis in a new patient cohort in order to identify additional biomarkers which may have been discounted due to false negative data.

Five miRNAs from the original 32 panel were selected for reanalysis in a new patient cohort comprising of 18 GBM patient samples and 18 control patient samples. MiR-19a, miR-19b and miR-17-5p were selected for reanalysis as they are part of the miR-17~92 cluster of miRNAs which also comprises of miR-20a and miR-92a. MiR-29c was selected for reanalysis as it has not been identified in the serum of glioblastoma patients previously. Power analysis was performed for miR-17-5p and miR-101 using the standard deviations determined in Section 4.2.4 (Table 4.7) to determine the sample size required for at least 80 % power. MiR-19a and miR-19b were not detected in the identification

cohort in Section 4.2.4, consequently power analysis could not be performed therefore a patient cohort consisting of 18 GBM samples and 18 control samples was utilised to perform the reanalysis. MiR-19a was not detected in the serum of glioblastoma patients following reanalysis. Expression of the remaining four reanalysed miRNAs was not significantly different (p > 0.05) between glioblastoma patients and matched controls (Figure 4.20 further analysed in Appendix 4).

Table 4.7 Power analysis for miR-17-5p and miR-101 reanalysis

MiRNA	SD	Sample Size Required for 80 % Power	Power of Reanalysis cohort of 18 patient samples
miR-17-5p	0.37	4	99 %
miR-101	0.55	6	99 %

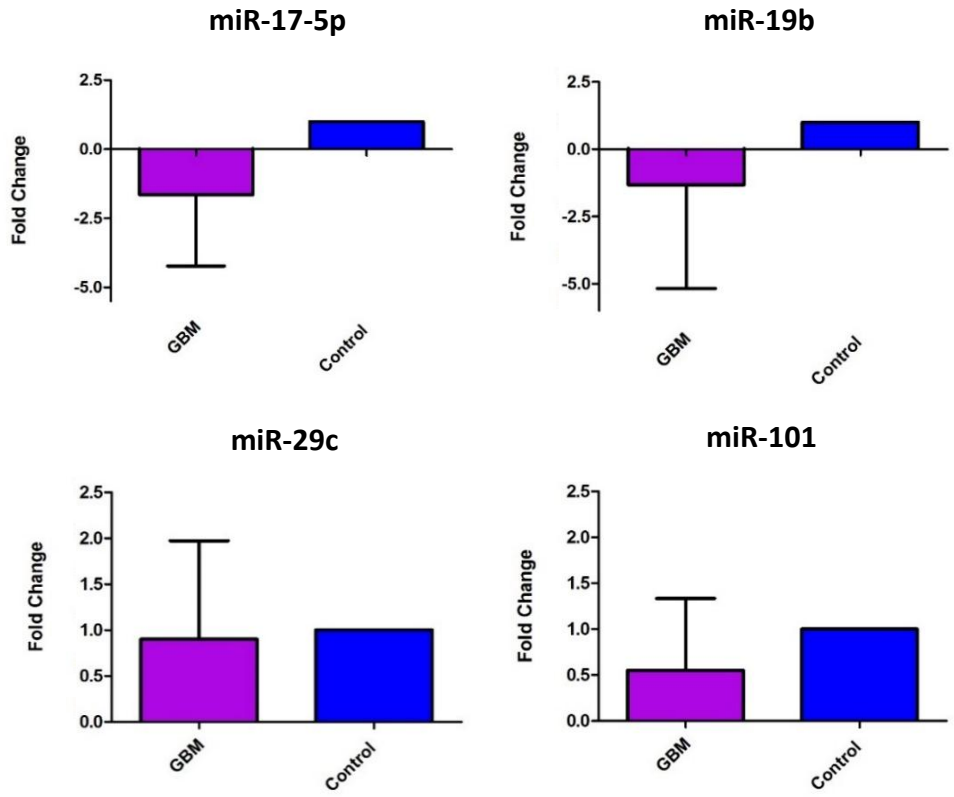


Figure 4.20 Expression of four re-analysed miRNAs in glioblastoma patient serum. Four of the five re-analysed miRNAs showed no significant difference in expression in GBM patient serum compared to matched controls. MiR-19a was undetected. Data shown as mean of replicates (n = 18 per group) plus SD, MiR-29c and 101 analysed by unpaired student's t-test, miR-17-5p and 19b analysed by Mann Whitney U test, p > 0.05.

4.2.7 Prognostic Potential of Serum MiRNA Biomarkers

Following the identification of four significantly dysregulated miRNAs, survival of the patients utilised in both the identification and validation panel study were analysed. Patient survival data for the samples used in this study were obtained from the BTNW tissue bank. Initially, overall survival was plotted for all glioblastoma patients used regardless of age or gender, 78 glioblastoma patients and 73 control patients were used in this study. Patients were then grouped by age and gender to determine any differences in overall survival between these cohorts. Following analysis of patient variable such as age and gender, patients were subsequently grouped by expression of the miRNA biomarkers validated in Sections 4.2.5. Analysis of miR-20a in the serum of glioblastoma patients identified two groups of glioblastoma patients; those with at least a two fold increase in miR-20a and those without. Survival data for these patients was obtained and grouped by miR-20a expression to determine whether miR-20a was a prognostic indicator for glioblastoma patients.

Overall survival of glioblastoma patients compared to non-cancerous control patients was lower with the median overall survival for glioblastoma patients used in this study being 6.62 months (Figure 4.21). All individuals within the control group were alive during this study. When grouped by age, median survival for patients aged between 20 and 39 years was 8.88 months. For patients aged between 40 and 59 years, median survival was 6.53 months and for patients aged over 60 years median survival was 3.36 months (Figure 4.22). No significant difference in survival was observed between the 20-39 and 40-59 age groups ($p > 0.05$). Survival for the 40-59 age group was significantly different ($p < 0.05$) when compared to the 60+ age group and for the 20-39 age group when compared to the 60+ group ($p < 0.001$). When grouped by gender no significant difference in survival was observed ($p > 0.05$). Male patients had a median survival of 5.88 months whereas female patients had a median survival of 7.98 months (Figure 4.23).

Analysis of survival for glioblastoma patients over the age of 60 years from the biomarker identification cohort grouped by miR-34a expression showed that patients with a low expression of miR-34a had a median survival of 10.6 months and patients with a high

expression of miR-34a had a median survival of 8.78 months (Figure 4.24). Of the six patients analysed in the identification cohort, four exhibited a greater than two fold change of miR-34a, with one patient still living and two patients exhibited a less than two fold change, with one patient still living. Median survival between patients with and without a two fold change in miR-34a expression in the identification cohort was not significantly different ($p > 0.05$).

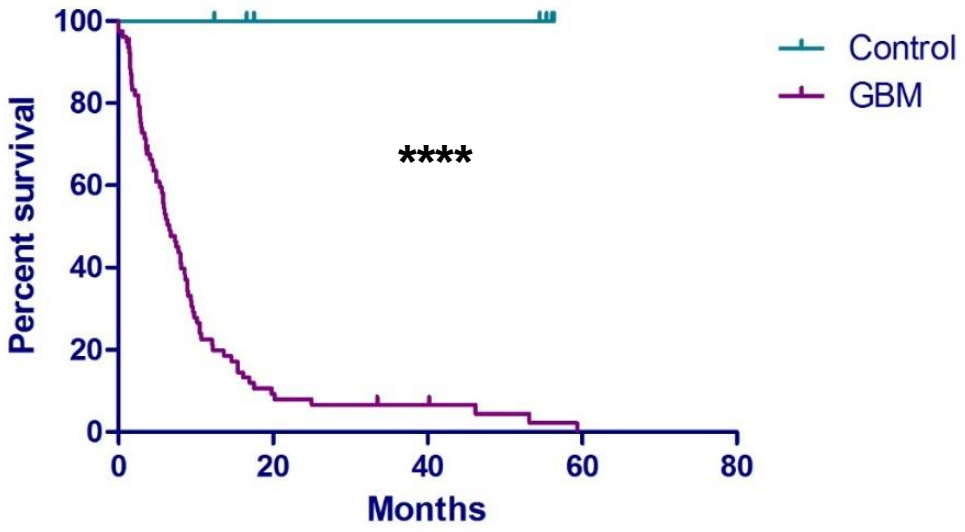


Figure 4.21 Kaplan-Meier survival curve of glioblastoma (GBM) patients compared to patients without glioblastoma (Control). Median survival of 78 GBM patients used in this study was 6.62 months. Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Survival was significantly different between the groups determined by Mantel-Cox test, ****$p < 0.0001$.

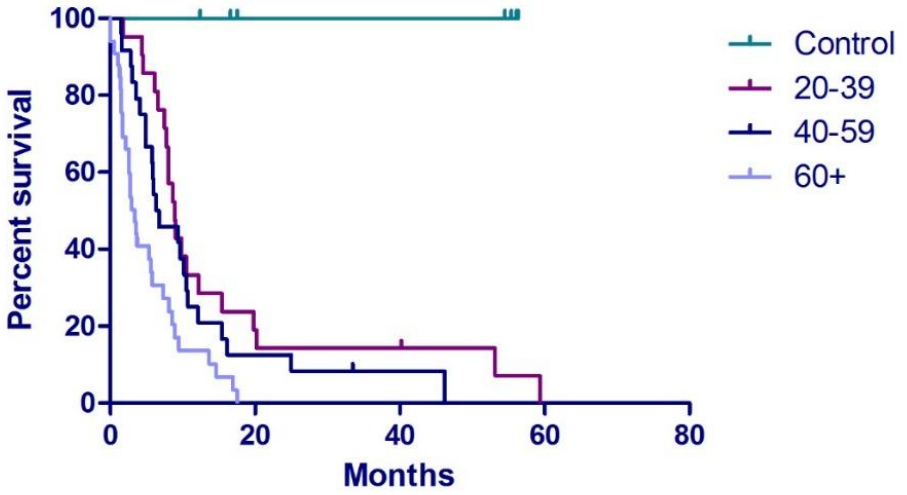


Figure 4.22 Kaplan-Meier survival curve of glioblastoma (GBM) patients grouped by age. Median survival of GBM patients grouped by age was 8.88 months for patients aged between 20 and 39 years, 6.53 months for patients aged between 40 and 59 years and 3.36 months for patients aged over 60 years. Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Survival was significantly different between the 20-39 and 60+ age groups ($p < 0.001$) and the 40-59 and 60+ age groups ($p < 0.05$) but not the 20-39 and 40-59 age groups ($p > 0.05$) determined by Mantel-Cox test.

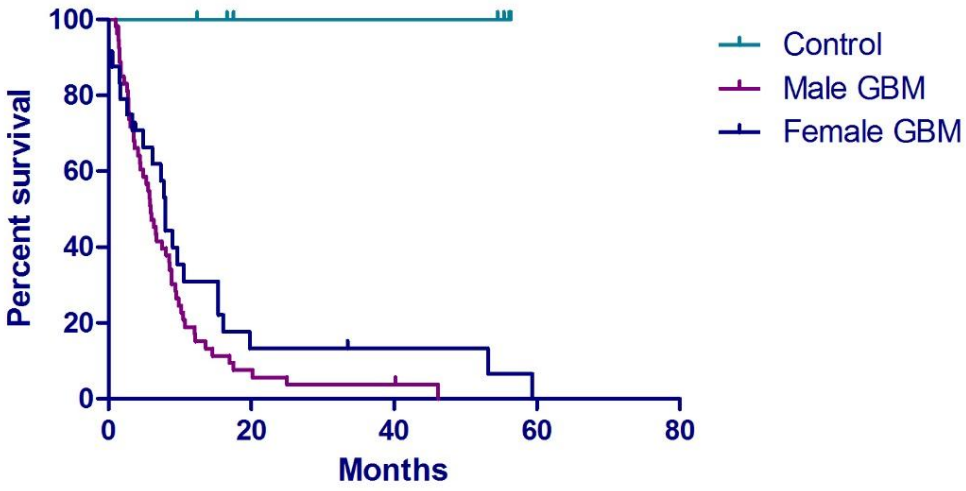


Figure 4.23 Kaplan-Meier survival curve of glioblastoma (GBM) patients grouped by gender. Median survival for male GBM patients (n = 54) was 5.8 months compared to 7.98 months for female GBM patients (n = 24). Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Survival was not significantly different determined by Mantel-Cox test, $p > 0.05$.

In the validation cohort, five patients exhibited a greater than two fold increase of miR-34a expression and exhibited a median survival of 4.85 months (Figure 4.25). Three patients exhibited a less than two fold change in miR-34a expression in the validation cohort and had a median survival of 7.32 months (Figure 4.25). Median survival was not significantly different ($p > 0.05$) between patients with and without a two fold change in miR-34a expression.

Combined analysis of 60+ glioblastoma patients in both the identification and validation cohorts showed that the nine patients with a greater than two fold increase in miR-34a expression exhibited a median survival of 9.49 months, the five patients with a less than two fold change in miR-34a expression showed a median survival of 8.05 months (Figure 4.26). Survival was not significantly different ($p > 0.05$) between patients with and without a twofold change in expression.

Analysis of patient survival by miR-92a expression showed no significant difference ($p > 0.05$) in expression between patients with a greater than two fold change in expression and those without.

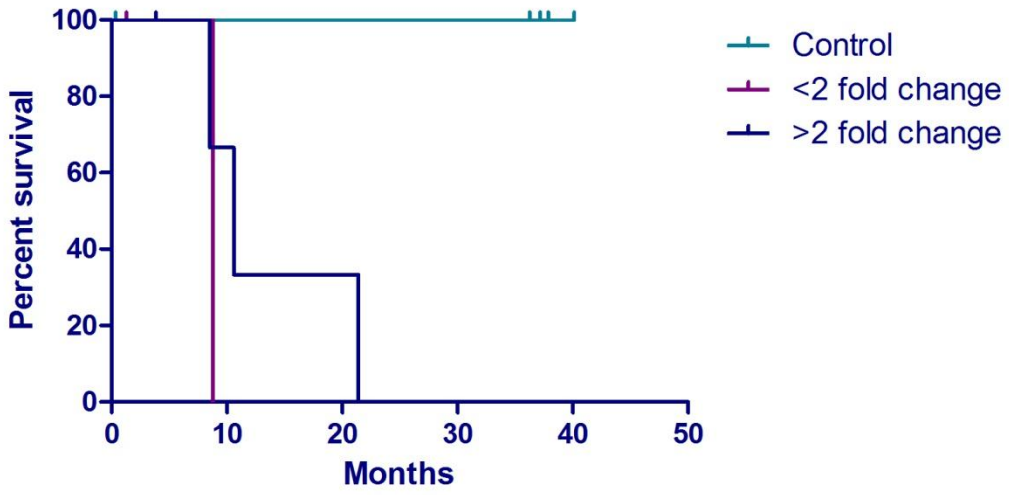


Figure 4.24 Kaplan-Meier survival curve of glioblastoma patients over the age of 60 years grouped by miR-34a expression. Median survival for patients with a high expression of miR-34a was 10.6 months (n = 4). Median survival for patients with a low expression of miR-34a was 8.78 months (n = 2). Survival was not significantly different between groups determined by Mantel-Cox test, $p > 0.05$

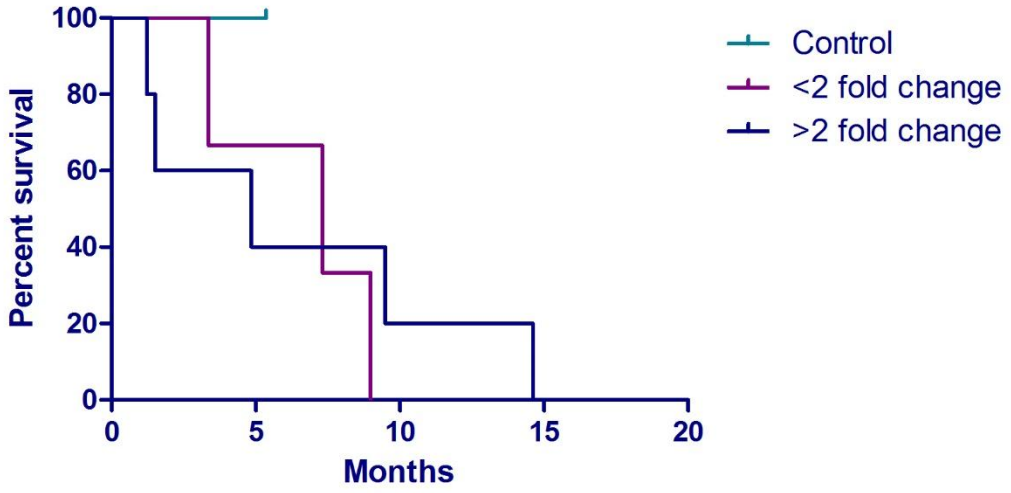


Figure 4.25 Kapan-Meier survival curve of 60+ glioblastoma patients in the validation cohort grouped by miR-34a expression. Median survival for patients in the validation cohort with a greater than two fold change of miR-34a was 4.85 months (n = 5). Median survival for patients with a less than two fold change in miR-34a expression was 7.32 months (n= 3). Survival was not significantly different between groups determined by Mantel-Cox test, $p > 0.05$

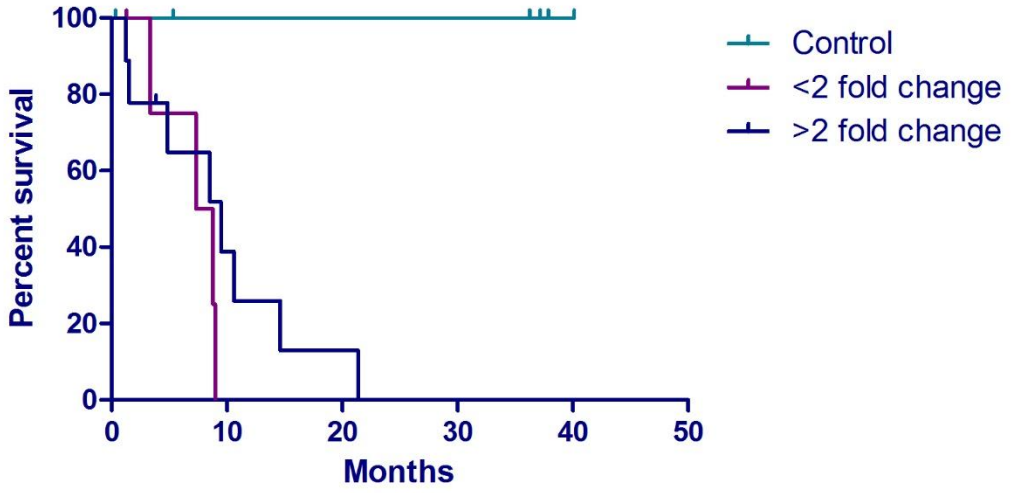


Figure 4.26 Kaplan-Meier survival curve of combined identification and validation patient cohorts over the age of 60 years grouped by miR-34a expression. Median survival for patients with a two fold change in miR-34a expression was 9.49 months (n = 9). Median survival for patients with a less than two fold change in miR-34a expression was 8.05 months (n = 5). Survival was not significantly different between groups determined by Mantel-Cox test, $p > 0.05$

Male glioblastoma patients with an increase in expression of miR-92a had a median survival of 9.55 months and those with no change in miR-92a expression had a median survival of 6.30 months (Figure 4.27). From the nine male patients whose samples were used in the identification cohort, six exhibited a greater than two fold increase and three exhibited a less than two fold change in expression. Analysis of male glioblastoma patients in the validation cohort showed 28 patients with a greater than two fold increase of miR-92a with a median survival of 5.67 months (Figure 4.28). Eight patients exhibited a less than two fold change in expression and had a median survival of 5.85 months, therefore survival was not significantly different ($p > 0.05$) between patients with and without a two fold change in miR-92a expression (Figure 4.28). Combined analysis of both the identification and validation cohorts for miR-92a expression showed no significant difference ($p > 0.05$) in expression between those with a two fold increase of miR-92a and those without (Figure 4.29).

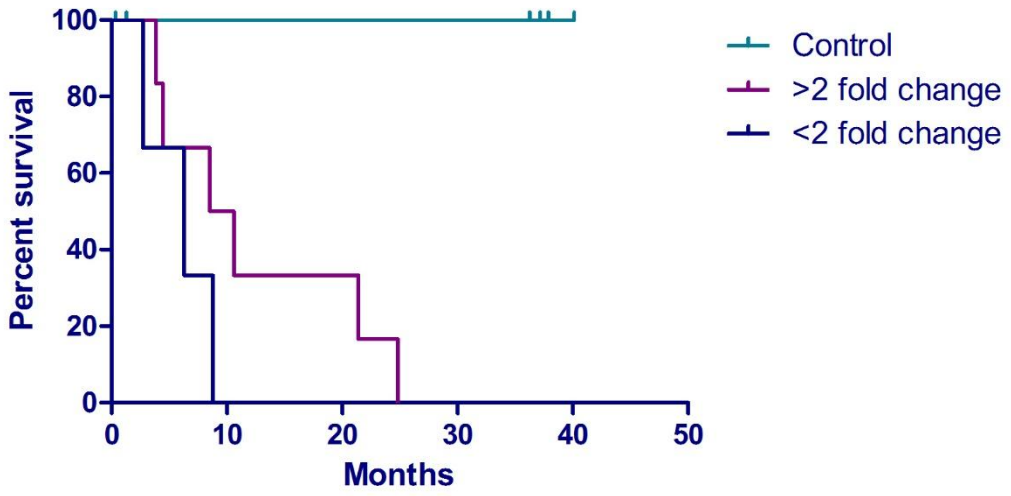


Figure 4.27 Kaplan-Meier survival curve of male glioblastoma patients grouped by miR-92a expression. Median survival for male glioblastoma patients with a high expression of miR-92a was 9.55 months (n = 6). Median survival for patients with a low expression of miR-92a was 6.30 months (n = 3). Survival was not significantly different determined by Mantel-Cox test, $p > 0.05$.

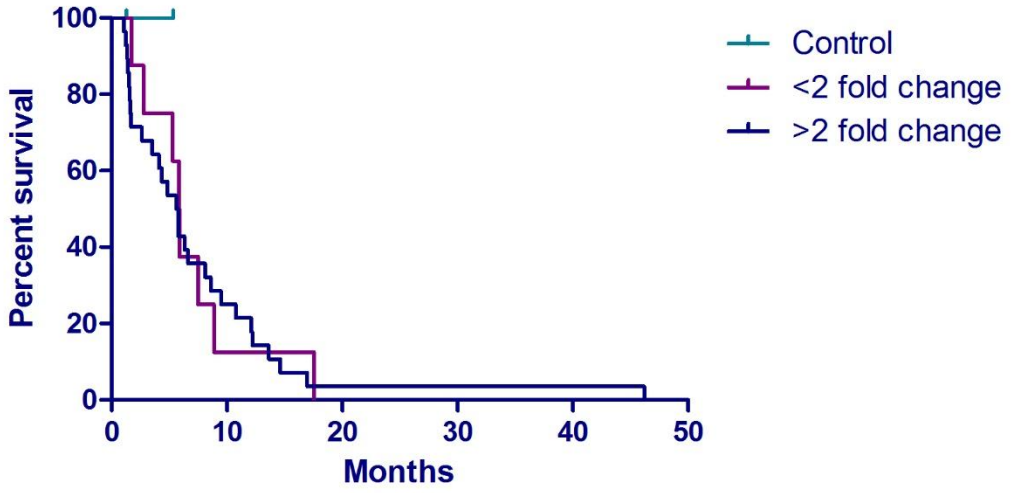


Figure 4.28 Kaplan-Meier survival curve of male glioblastoma patients in the validation cohort grouped by miR-92a expression. Median survival for male glioblastoma patients with a greater than two fold change in miR-92a was 5.67 months (n = 28). Median survival for male glioblastoma patients with a less than two fold change in miR-92a was 5.85 months (n = 8). Survival was not significantly different determined by Mantel-Cox test, $p > 0.05$.

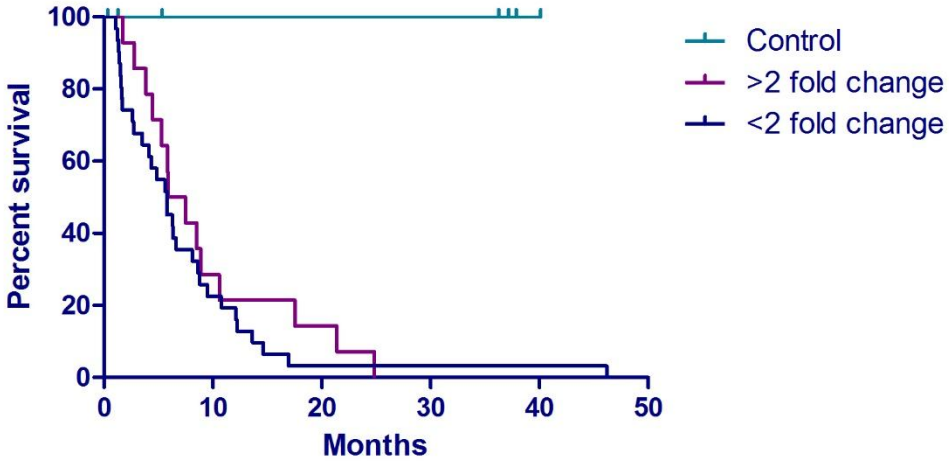


Figure 4.29 Kaplan-Meier survival curve of male patients from both the identification and validation cohorts grouped by miR-92a expression. Median survival of male glioblastoma patients with a greater than two fold change in miR-92a expression had a median survival of 6.68 months (n = 34). Median survival of male glioblastoma patients with a less than two fold change in miR-92a expression had a median survival of 5.75 months (n = 11). Survival was not significantly different determined by Mantel-Cox test, $p > 0.05$.

The patient cohorts used in the initial biomarker identification study and validation study both exhibited a reduced expression of miR-30c. The reduction in expression however, was greater in the validation study cohort than in the identification study cohort, therefore the median survival for the two cohorts was compared (Figure 4.30). Median survival for patients in the two cohorts was not significantly different ($p > 0.05$) with patients in the identification cohort having a median survival of 30.89 months and patients in the validation cohort having a median survival of 20 months (Figure 4.30).

Patients used in the initial biomarker identification study with an increase in miR-20a had a median survival of 21.39 months compared to 4.45 months for patients with no change in miR-20a expression $p < 0.001$ (Figure 4.31). Patients in the validation cohort with an up-regulation of miR-20a had a median survival of 9.49 months compared to 1.65 months for patients with no change in miR-20a expression $p < 0.001$ (Figure 4.32A). Combined survival analysis of patients from the initial biomarker identification phase and patients from the validation cohort showed that patients with an increase of miR-20a had a median survival of 10.12 months compared to a median survival of 3.04 months $p < 0.001$ for those with no change in miR-20a expression (Figure 4.32B).

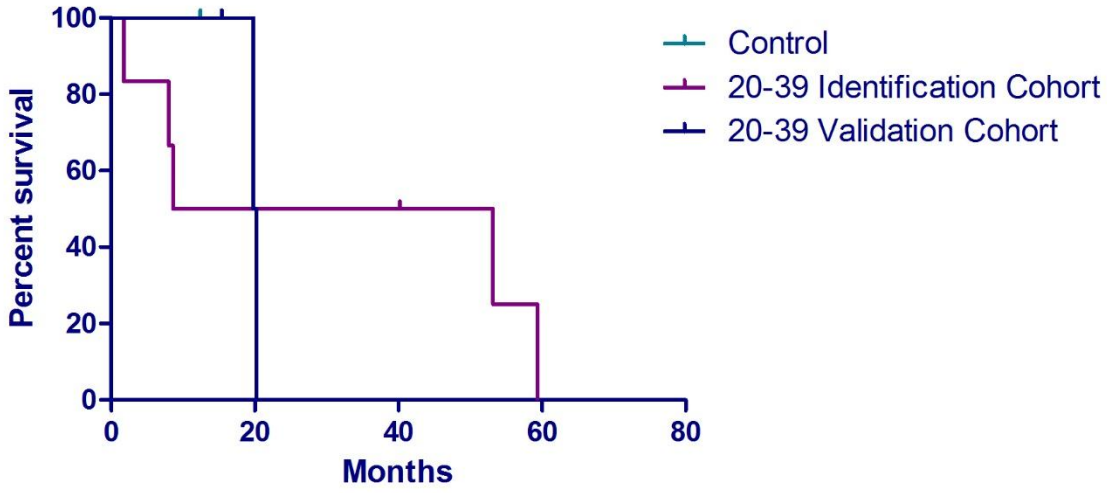


Figure 4.30 Kaplan-Meier Survival Curve of glioblastoma patients aged between 20-39 years grouped by 30c expression. Mean fold change of miR-30c in the serum of glioblastoma patients aged between 20-39 years was 0.17 and these patients exhibited a median survival of 30.89 months (n = 9). Mean fold change for patients in the validation cohort was -1.36 and these patients exhibited a median survival of 20 months (n = 3). Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Median survival between the two cohorts was not significant, determined by Mantel-Cox test, $p > 0.05$.

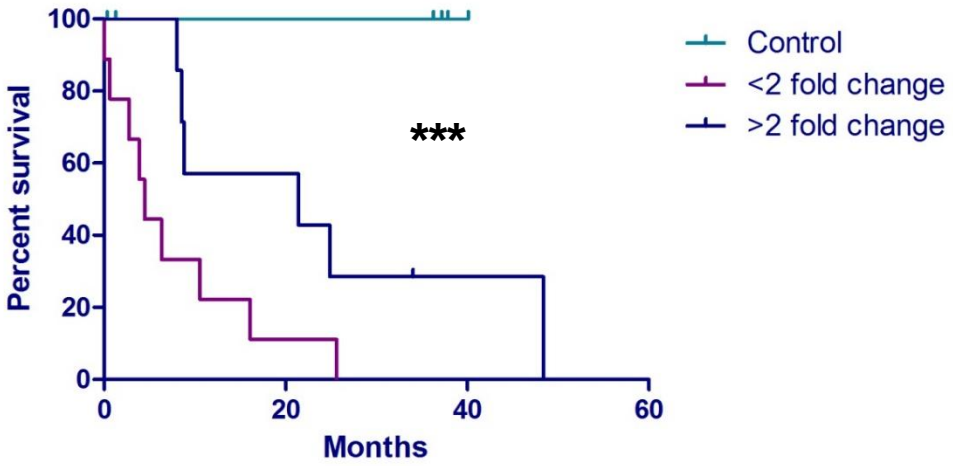


Figure 4.31 Kaplan-Meier survival curve of glioblastoma patients grouped by miR-20a expression. Median survival for GBM patients with a 2-fold or greater increase of miR-20a expression was 21.39 months (n = 9). Median survival for patients with less than a 2-fold change in miR-20a expression was 4.45 months (n = 9). Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Survival was significantly different between groups determined by Mantel-Cox test, ***$p < 0.001$.

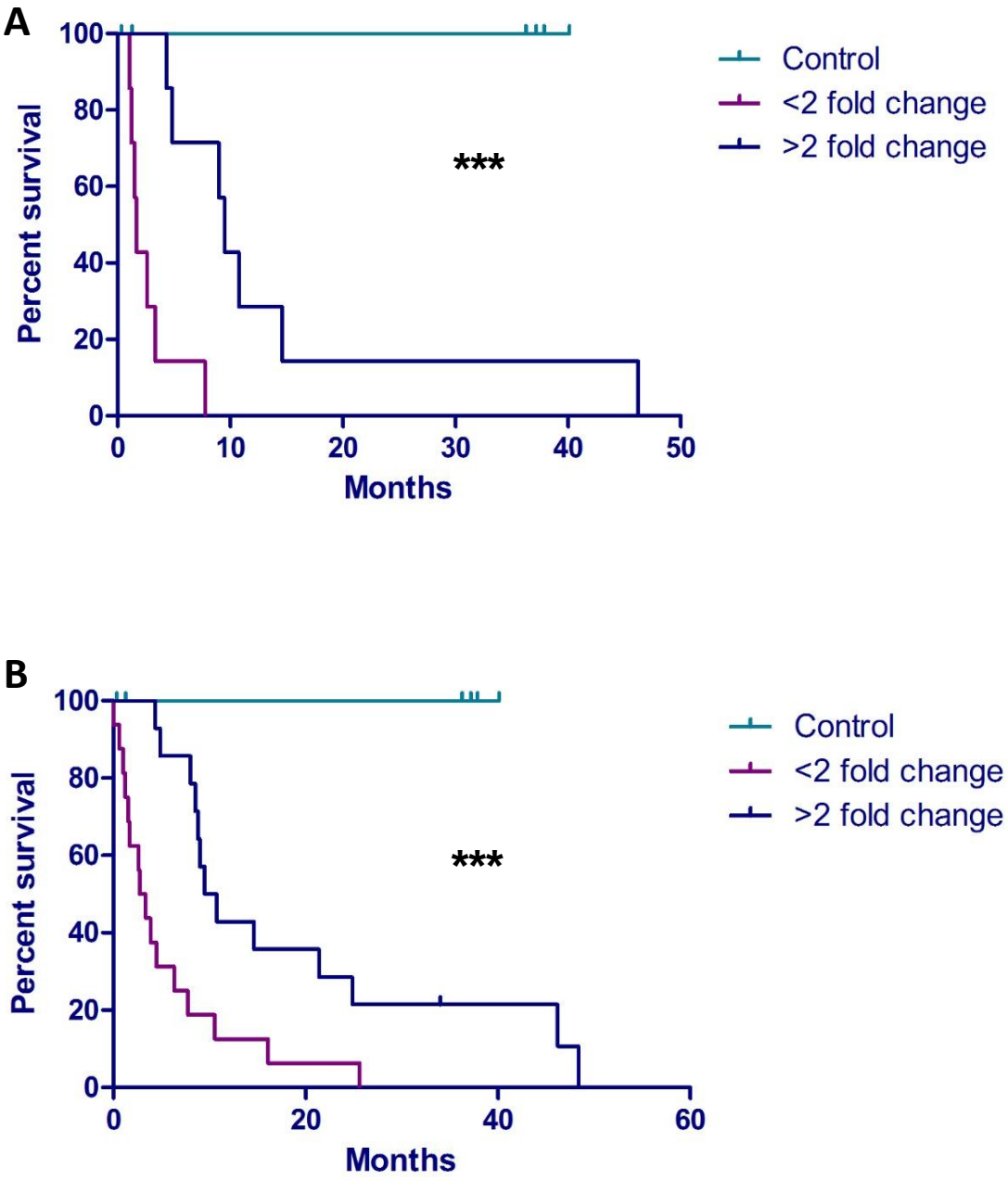


Figure 4.32 Kaplan-Meier survival curve of glioblastoma patients grouped by miR-20a expression. A) Median survival of GBM patients in the validation set with a greater than two fold increase of miR-20a expression was 9.49 months compared to 1.65 months for patients with no change in miR-20a expression. B) Combined survival data for patients from both initial biomarker identification and validation cohorts displayed a median survival of 10.12 months for patients with a greater than two fold increase of miR-20a expression and 3.04 for patients with no change in miR-20a expression. Steps down indicate the death of a patient, dashes indicate patients alive at the time of this study. Survival was significantly different between groups determined by Mantel-Cox test, ***$p < 0.001$.

4.2.8 Correlation between MiR-34a and Patient Age

MiR-34a expression has previously been shown to have a correlation with the age of individuals (Li et al., 2011b, Sawada et al., 2014). The correlation between miR-34a and age was analysed in the serum samples used in this study to elucidate whether a similar trend in expression and age was observed. In order to determine the expression of miR-34a in the control samples as well as the glioblastoma samples, delta Ct values were used for the analysis of miR-34a expression and age.

Analysis of miR-34a expression in control serum showed no significant difference in miR-34a expression in patients over the age of 60 years compared to those aged between 40 and 59 years (Figure 4.33). MiR-34a was not detected in non-cancerous serum from individuals aged between 20 and 39 years. MiR-34a was increased in the sera of glioblastoma patients aged over 60 years compared to patients aged between 40 and 59 years (Figure 4.33). MiR-34a was not detected in the serum of glioblastoma patients aged between 20 and 39 years. Expression of miR-34a was higher in glioblastoma patients over the age of 60 years compared to age-matched control patients. Expression of miR-34a in glioblastoma patients aged between 40 and 59 years was not significantly different ($p > 0.05$) when compared to age-matched controls (Figure 4.33).

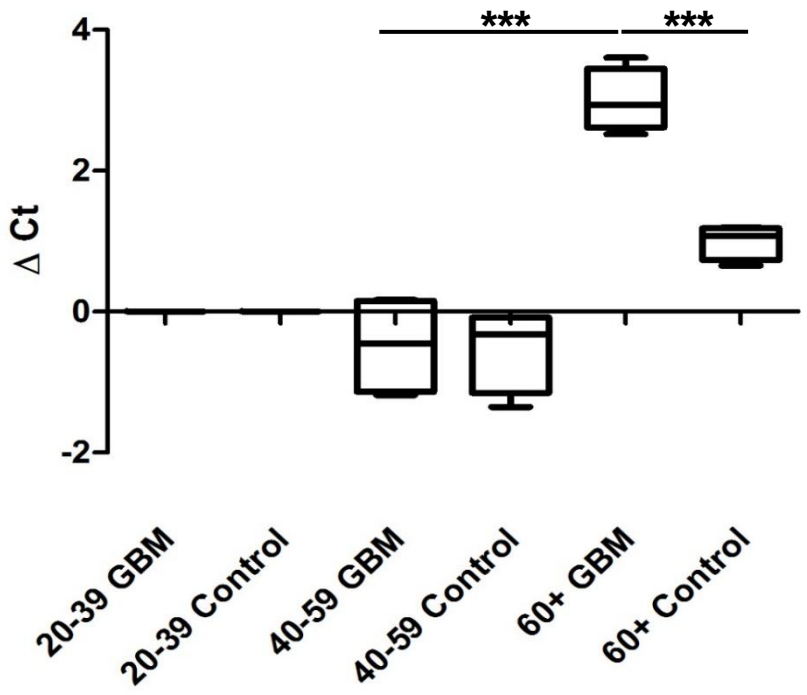


Figure 4.33 Expression of miR-34a by age in glioblastoma and control patient serum. MiR-34a was significantly higher in glioblastoma patients over the age of 60 years compared to the 40 and 59 group. MiR-34a was significantly higher in glioblastoma patients over the age of 60 years compared to age matched controls. Data analysed by one way ANOVA with Tukey *post-hoc* test. Significance between groups indicated by a solid black line, ***$p < 0.001$.

4.2.9 Relationship between MiR-92a and MiR-20a Expression

MiR-92a and 20a are part of the 17~92 cluster of miRNAs which are located in the same region of the genome and transcribed together (Mendell, 2008), therefore the expression of these two miRNAs was analysed together in the serum of each glioblastoma and control patient to determine whether there was a correlation between the expression levels. Analysis of miR-92a and 20a in serum from control patients (Figure 4.34A) and patients with glioblastoma (Figure 4.34B) showed no correlation in expression.

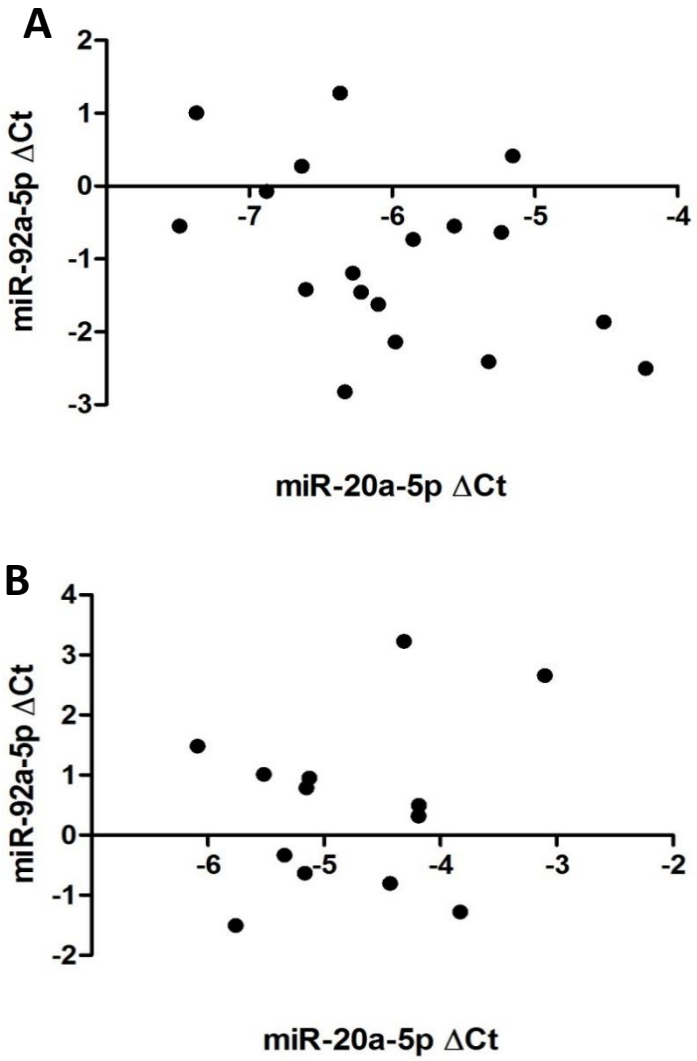


Figure 4.34 Relationship between miR-20a and miR-92a expression. A) Analysis of serum miR-20a and miR-92a expression in individuals without glioblastoma showed no correlation. B) Analysis of serum miR-20a and miR-92a expression in glioblastoma patients showed no correlation. Data analysed by Pearson correlation, A) $p > 0.05$ (r = 0.19), B) $p > 0.05$ (r = 0.06).

4.3 Discussion

The miRNA expression profiles obtained in this chapter exhibited differences in miRNA expression between genders and age groups of glioblastoma patients, highlighting the importance of considering these variables when investigating miRNA biomarkers for disease, including glioblastoma. Analysis of miRNA expression using the miScript brain cancer array identified miR-191-5p alone to be up-regulated in both male and female glioblastoma patients in all three age groups. MiR-191-5p has previously been identified to be up-regulated in glioblastoma cell lines (Ciafrè et al., 2005) however, there is no current research investigating the role of miR-191-5p in glioblastoma. MiR-191-5p is part of the miR-191/425 cluster which is expressed in an intron of the *DALRD3* gene with which it is co-expressed (Kulshreshtha and Nagpal, 2014). Altered expression of miR-191 has been observed in a number of cancer types including lung, liver, prostate and breast cancer, however whether expression is up or down-regulated depends on the cancer type (Kulshreshtha and Nagpal, 2014).

Five miRNAs were identified which displayed significantly altered expression in the sera of glioblastoma patients: miR-20a, miR-30c, miR-34a, miR-92a and miR-150. MiR-34a expression was up-regulated in the serum of glioblastoma patients over the age of 60 years. MiR-34a has been found to be down-regulated in glioblastoma tissue and is a target of p53, a known tumour suppressor (Okada et al., 2014). MiR-34a targets c-Met, Notch-1 and Notch-2 in glioblastoma tissue to inhibit proliferation and invasion (Li et al., 2009). MiR-34a expression in the brain has previously been found to increase with age (Li et al., 2011b) which corroborated the data observed in this chapter. Analysis of ΔCt values of both control and glioblastoma patient serum showed an increase in expression of miR-34a with age. Further to the correlation between miR-34a expression and age, the expression of miR-34a was observed to be higher in glioblastoma patients compared to age matched control patients in which patients over the age of 60 years with glioblastoma exhibited a higher expression of miR-34a compared to age-matched controls. Glioblastoma patients aged between 40 and 59 years displayed a similar expression of miR-34a compared to age-matched controls suggesting the presence of the glioblastoma further promoted the expression of miR-34a in the serum of patients over the age of 60 years.

MiR-34a has not previously been identified in the serum of glioblastoma patients or any other disease, therefore the up-regulation of miR-34a in this study cannot be compared to other studies. A similar difference in miRNA expression between tissue and biofluid samples was demonstrated in a previous study investigating miR-128 expression which was found to be up-regulated in the blood of glioblastoma patients but was down-regulated in glioblastoma tissue (Roth et al., 2011). Analysis of miRNA expression in the blood of other diseases such as multiple sclerosis and melanoma have also identified an inverse correlation between circulating expression and tissue expression (Roth et al., 2011). The difference in expression between the serum up-regulation observed in this thesis and the tissue down-regulation of miR-34a observed in previous studies could be due to a number of factors such as selective exosomal packaging (Chen et al., 2012), or competitive interactions between glioblastoma cells and neighboring non-cancerous cells in the tumour microenvironment (Kosaka et al., 2012).

Non-cancerous epithelial cells can prevent or reduce the growth of transformed neighbouring cells by the release of autocrine and paracrine factors as a means of cell growth homeostasis in a process known as cell competition (Kosaka et al., 2012). Disruption of this homeostatic cell competition process has been proposed as a factor which can contribute to tumour initiation. Kosaka *et al.* (2012) demonstrated that non-cancerous epithelial prostate cells secreted tumour suppressor miRNAs as anti-proliferative signals which were able to attenuate proliferation of prostate cancer cells. Although non-cancerous cells have been shown to attenuate proliferation of transformed cells by the secretion of anti-proliferative miRNAs, cancerous cells can combat these signals by impairing the process of exosome uptake to prevent the incorporation of tumour suppressive miRNAs (Kosaka et al., 2012). MiR-34a exerts anti-proliferative effects by targeting cell cycle regulators such as Notch-1 (Li et al., 2011a), and could potentially be released by neighbouring epithelial cells in the brain in response to the presence of glial tumours. Impaired uptake of exosomes containing miR-34a by glioblastoma cells could lead to an enrichment of these exosomes containing miR-34a and subsequently the increase in expression of this miRNA in glioblastoma patient serum samples.

MiR-92a expression was high in the serum of male glioblastoma patients whilst female glioblastoma patients exhibited no change in expression. The difference in expression between genders may be due to the location of multiple miR-92a sequences in the genome. MiR-92a-1 is found within the 17~92 cluster of miRNAs on chromosome 13 and miR-92a-2 is found within the paralogous cluster 106~363 on the X chromosome, both miR-92a-1 and 92a-2 share the same miR-92a-3p sequence. Females have two X chromosomes and males have one, to maintain equilibrium of gene expression between male and females, the mechanism of X-chromosome inactivation (XCI) occurs during embryonic development in which one X chromosome is inactivated at random (Pinheiro et al., 2011). XCI can be 'skewed' where the X-chromosome containing a detrimental mutation can be preferentially silenced, this has been observed for recessive X-linked immune disorders, where females are carriers for the disorder and do not exhibit symptoms (Pinheiro et al., 2011). This mechanism can also affect miRNAs found on the X chromosome which are also susceptible to skewing and therefore could account for differences in miRNA expression between genders. Mutations in one X-chromosome, therefore, could cause the up-regulation of miR-92a-2 to be silenced by XCI in females, whereas males are hemizygous, thus the same mutation cannot be silenced leading to an up-regulation of miR-92a-3p. Furthermore, as males are hemizygous for X-linked mutations in both protein encoding genes and miRNA, this may partially explain the difference in gender susceptibility to cancer (Pinheiro et al., 2011), including glioblastoma, and differences in survival as observed in this study.

MiR-20a showed differential expression between glioblastoma patients in the initial identification cohort. One subset exhibited a more than two fold increase in expression compared to controls however the other subset of patients exhibited no change in expression. The difference in expression between the two groups of glioblastoma patients could indicate a difference in glioblastoma subtype. Glioblastoma subtypes differ in the profile of mutations present as well as miRNA expression, which could subsequently affect miRNA expression as observed in this study. Analysis and grouping of glioblastoma data in The Cancer Genome Atlas (TCGA) based on neural differentiation for identifying glioblastoma subtypes led to identification of miR-20a expression in the oligoneural precursor expression profile (Kim et al., 2011). Patients

used in this current study with an up-regulation of miR-20a could be of the oligoneural precursor subtype and those without an up-regulation could be of a different glioblastoma subtype. The use of miRNA biomarkers during diagnosis to identify glioblastoma subtypes could improve treatment decisions based on the response of a particular glioblastoma subtype due to the specific molecular profile.

MiR-30c was observed to have a significantly lower expression in the serum of glioblastoma patients between the ages of 20 and 39 years compared to matched control patients. A decrease in miR-30c expression in the serum of glioblastoma patients has not previously been reported however, an increase in miR-30c expression has been reported in TRAIL-resistant glioblastomas (Quintavalle et al., 2012).

Validation of the five miRNA biomarkers identified in Section 4.2.4 using sample numbers determined by power analysis confirmed that four of the five miRNAs showed a similar trend in the second cohort in Section 4.2.5. MiR-92a was shown to have a high expression in male glioblastoma patient serum by a similar fold change in the validation cohort compared to the initial study. MiR-34a and miR-20a were increased in the validation cohort as observed in the initial study, however both miRNAs showed a smaller increase in the validation study compared to the initial study. The mean expression of miR-34a in the initial study was approximately a three-fold increase, in the validation study the mean expression of miR-34a was approximately a two fold increase however, the standard deviation of miR-34a expression in the validation study shows that there were certain samples within the cohort which showed a similar expression to samples within the initial study. MiR-20a showed the greatest variation in expression between the initial and validation groups. This could be due to the variation in expression of different proteins which may target miR-20a between different subtypes of glioblastoma. Patients with decreased expression in miR-30c in the identification cohort exhibited a median decrease of 0.5 fold however patients in the validation cohort exhibited a median decrease of -1.05 fold. The variation in median fold decrease of miR-30c expression could be due to variables other than glioblastoma such as drug treatments or the presence of other diseases which may affect miRNA expression in either the glioblastoma patient cohort or the control patient cohort.

Out of the four validated miRNAs, miR-20a showed a significant difference in median survival times between patients with an increase in expression of miR-20a, compared to those without. Patients used in the initial biomarker identification cohort with an increase of miR-20a had a median survival time of 21.39 months compared to a median survival time of 4.45 months for patients without an increase in miR-20a. Patients in the validation cohort with an increase of miR-20a also exhibited a better prognosis, 9.49 months median survival, compared to those with no change in expression, 1.65 months median survival. MiR-20a has the potential, therefore, to be a prognostic miRNA biomarker for glioblastoma patients, which could be utilised for improved treatment strategies.

Analysis of survival data for the patients used in this study identified that age and gender both had an effect on the median patient survival time. Overall, the median survival time for glioblastoma patients used in this study was 6.62 months, this is lower than the median survival time of 9 months generally observed for glioblastoma patients (Alqallaf et al., 2014). When grouped by age, glioblastoma patients aged between 20 and 39 years had the best median survival time of 8.88 months. Patients aged between 40 and 59 years had a median survival time of 6.53 months and patients over the age of 60 years had the worst median survival time of 3.36 months. Glioblastoma patients over the age of 60 years are often difficult to treat due to their reduced ability to cope with aggressive therapies (Arvold and Reardon, 2014). Median survival times for male and female glioblastoma patients were 5.88 and 7.98 months, respectively, but this difference was not statistically significant.

MiR-20a and 92a-3p are expressed within the miR-17~92 cluster which comprises of a group of six miRNAs; miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1, which are located on chromosome 13 and are transcribed together (Olive et al., 2010). In glioblastoma the 17~92 cluster is up-regulated and plays a role in apoptosis and proliferation. In this study, only two of the six miRNAs transcribed in this cluster were found to have altered expression, this suggests that the miRNAs within this cluster may be subject to, currently unknown, specific post-transcriptional regulation resulting in the varied expression profiles (Mendell, 2008). Five of the six miRNAs which make up this cluster were identified in the 32 miRNA panel in Section 4.2.4 however validation of the 32 miRNA indicated that the expression of miR-17, miR-19a and miR-19b was

not significantly different in the serum of glioblastoma patients. Furthermore, additional analysis of these miRNAs to determine false negatives again showed no significant difference in expression between glioblastoma patient serum and matched controls. MiRNAs can be subject to post-transcriptional regulation by selective processing to produce mature miRNAs, in this manner miRNA genes may be transcribed and partially processed to produce the pre-miRNA form but further cleavage and processing does not occur to produce the active mature miRNA which can go on to target mRNA (Obernosterer et al., 2006). It has been shown that for some miRNAs high levels of pre-miRNA can be detected in cells but little or no expression of the mature form is seen. These pre-miRNA appear to be localised in the nucleus suggesting that these miRNA genes are transcribed, processed into pre-miRNA but are not subsequently exported to the cytoplasm for further processing (Lee et al., 2008). Due to the location of miR-92a and miR-20a in the 17~92 cluster, expression of these two miRNAs in each individual patient sample was analysed to determine whether they were correlated. Expression of miR-92a and miR-20a was observed to have no correlation in both glioblastoma patient samples and non-cancerous control samples. This again suggests that although these miRNAs are transcribed together from the cluster, differential post-transcriptional regulation affects the expression of the mature miRNA forms. Conversely, miR-20a and 92a-3p may have correlated expression within the tissue of glioblastoma but this correlation may not extend to serum secretion, this could again be a result of selective exosomal packaging (Chen et al., 2012) which could result in differences in expression within the serum of these two miRNAs.

In summary, data from this chapter have identified that miRNA expression is dysregulated in the serum of glioblastoma patients with age and gender differences in expression. Four miRNAs, miR-20a, miR-30c, miR-34a and miR-92a were identified and found to be significantly increased in the serum of glioblastoma patients which were validated in a larger patient cohort. In addition to being dysregulated in the serum of glioblastoma patients, miR-20a was also found to have prognostic potential, where a subset of patients exhibiting a greater than two fold increase in expression showed better survival than those with no change, which could be linked to an oligoneural precursor subtype of glioblastoma. Expression of miR-20a was found to be inversely correlated with age, with younger glioblastoma patients exhibiting the highest

expression and older patients aged over 60 years exhibiting the lowest. Further investigation of miR-92a, found to be expressed at a higher level in male glioblastoma serum sample, could provide information on possible gender susceptibility to glioblastoma. MiR-34a expression was increased in the serum of glioblastoma patients aged over 60 years. Further analysis showed that miR-34a expression was correlated with age in both control and glioblastoma patient serum, suggesting that a combination of age and the presence of the tumour led to the up-regulation of this miRNA in the serum of older glioblastoma patients. As miR-34a has previously been identified as a tumour suppressor down-regulated in glioblastoma tissue, the finding that it is up-regulated in the serum of glioblastoma patients may suggest an alternative source of miRNA biomarkers in the circulation, other than the tumour itself. This led to the next aim of this thesis to elucidate the origin of miRNA dysregulation in the serum of glioblastoma patients.

5. Origin of MiRNA

5.2 Results

5.2.1 Identification of MicroRNA Biomarkers in Cell Lines Cultured in 10 % FBS

Following identification of four potential miRNA biomarkers, miR-20a, miR-30c, miR-34a and miR-92a in the serum of glioblastoma patients, U87MG and SVGp12 cells were cultured using the human serum cell culture model established in Chapter 3. The serum weaning study in Section 3.2.5 did not investigate the miRNA biomarkers validated in Section 4.2.5, therefore the serum weaning study was repeated to determine whether the serum biomarkers were expressed in the cell lines under standard culture conditions and to investigate the effects of serum weaning on expression. The investigation of serum miRNA biomarkers in immortalised cell lines provides a simplified model to help determine the origin of the miRNA biomarkers compared to *in vivo* systems in which there are alternative sources of miRNAs such as immune cells and cells from other tissues. As miRNAs are frequently part of positive feedback loops which promote further expression, the application of patient serum types in which each biomarker was up-regulated may affect the expression of the biomarker in recipient cells. Three of the four validated biomarkers identified in Section 4.2.5, miR-92a, miR-20a and miR-30c, have been found, in previous studies to be oncogenic (Mogilyansky and Rigoutsos, 2013, Quintavalle et al., 2012), therefore the release of these miRNAs in glioblastoma exosomes may also have a self-promoting function. To determine whether exosomes within the serum of glioblastoma patients could alter miRNA biomarker expression in recipient U87MG and SVGp12 cells, cells were weaned onto 10 % glioblastoma patient serum or 10 % control patient serum using new patient serum samples. Total RNA was extracted from within the cells following serum replacement to determine intracellular biomarker expression, and additionally exosomes were isolated from the spent media to determine extracellular biomarker expression. This would, in part, elucidate the effect of glioblastoma and control serum on recipient cell miRNA expression as well as changes in miRNA expression in secreted exosomes from the recipient cells themselves following culture in human serum.

Initial analysis was performed to determine the expression of the biomarkers in pooled triplicate patient serum samples alone compared to U87MG and SVGp12 cells cultured

in FBS before serum weaning. Cells were then cultured in glioblastoma and control patient serum corresponding to the age and gender category in which they were found to be dysregulated (Sections 4.2.4 and 4.2.5) to determine whether the dysregulation of these miRNAs in serum could have an effect on intracellular miRNA expression in recipient cells. Cells were cultured in serum from patients aged over 60 years to determine the effect on expression of miR-34a, male patient serum for miR-92a, glioblastoma patient serum for miR-20a and 20-39 years glioblastoma patient serum for miR-30c.

5.2.1.1 Expression of MiR-34a in 60+ Patient Serum Pools compared to U87MG and SVGp12 Cells Cultured in 10 % FBS

Prior to serum replacement, miR-34a expression in a 60+ age glioblastoma patient serum pool and a 60+ age control patient serum pool to be used for weaning was compared to the intracellular expression of miR-34a in U87MG and SVGp12 cells and spent media containing 10 % FBS. Comparison of miR-34a expression in the two serum pools showed that miR-34a expression was not significantly different ($p > 0.001$) in the 60+ age GBM patient serum pool compared to the 60+ age control patient serum pool (Figure 5.1). MiR-34a expression in both the 60+ age glioblastoma patient serum pool and the 60+ age control patient serum pool was not significantly different ($p > 0.001$) than intracellular miR-34a expression in U87MG and SVGp12 cells and spent media containing 10 % FBS (Figure 5.1). MiR-34a expression in U87MG cells cultured in 10 % FBS was not significantly different ($p > 0.001$) extracellularly in the spent media compared to intracellular expression (Figure 5.1). Similarly, intracellular miR-34a expression in SVGp12 cells cultured in 10 % FBS was not significantly different ($p > 0.001$) compared to extracellular expression in the spent media (Figure 5.1). Intracellular miR-34a expression in U87MG cells was not significantly different ($p > 0.001$) to intracellular miR-34a expression in SVGp12 cells, both cultured in 10 % FBS (Figure 5.1). Extracellular miR-34a expression in U87MG cells cultured in 10 % FBS was also not significantly different to extracellular miR-34a expression in SVGp12 cells cultured in 10 % FBS (Figure 5.1).

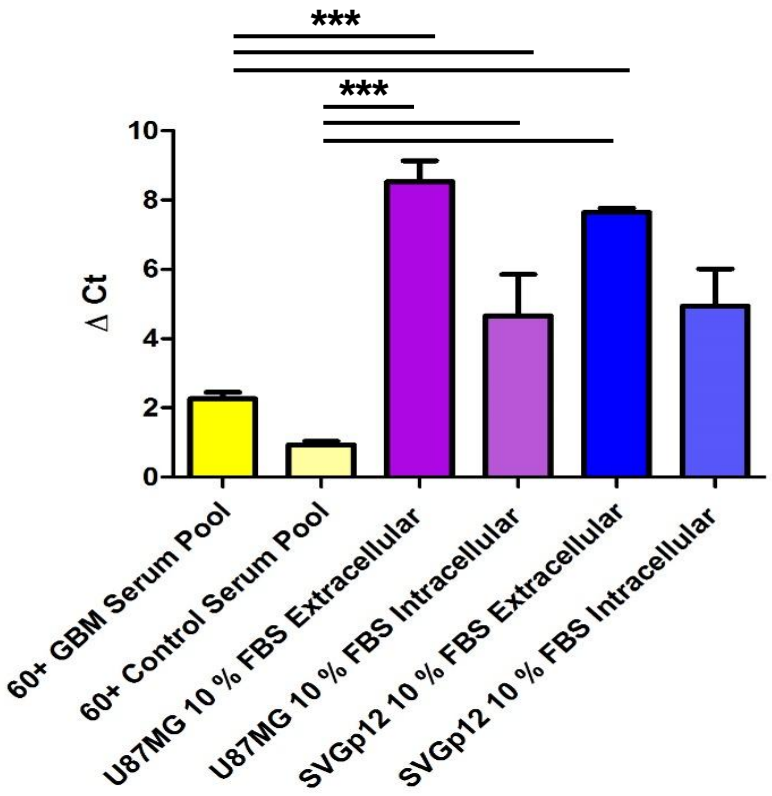


Figure 5.1 Comparison of miR-34a expression in 60+ GBM patient serum pool and 60+ control patient serum pool. MiR-34a expression in cell lines cultured in 10 % FBS was significantly higher than expression in the 60+ patient serum pools. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by one-way ANOVA with Tukey *post-hoc* test. Significance indicated between groups by a solid black line, ***$p < 0.001$.

5.2.1.2 Expression of MiR-34a in U87MG and SVGp12 cells following Serum Replacement with 60+ Patient Serum Pools

After establishing miR-34a expression in the 60+ age glioblastoma patient serum pool and 60+ control patient pool compared to U87MG and SVGp12 miR-34a expression when cultured in FBS, cells were weaned onto the 60+ age patient pools. The change in miR-34a expression following serum replacement was determined compared to cells cultured in FBS, all data were normalised using the cel-miR-39 spike in and then subsequently normalised to miR-34a expression in the corresponding serum pool. The normalisation of cell line expression to the corresponding serum pools ensured that expression of miR-34a produced by the cell themselves was determined. The culture of U87MG cells in both the 60+ age glioblastoma and 60+ age control patient serum pool led to a significant increase ($p < 0.001$) in intracellular miR-34a expression compared to those cultured in FBS (Figure 5.2). The increase in expression observed intracellularly in U87MG cells was not significantly different ($p > 0.001$) between those cultured in the

60+ glioblastoma patient serum and 60+ control patient serum (Figure 5.3). No significant change (p > 0.001) in miR-34a expression was observed extracellularly for U87MG cells cultured in either serum pool (Figure 5.2). The culture of SVGp12 cells in 60+ patient serum did not significantly alter miR-34a expression either intracellularly or extracellularly compared to those cultured in FBS (Figure 5.2).

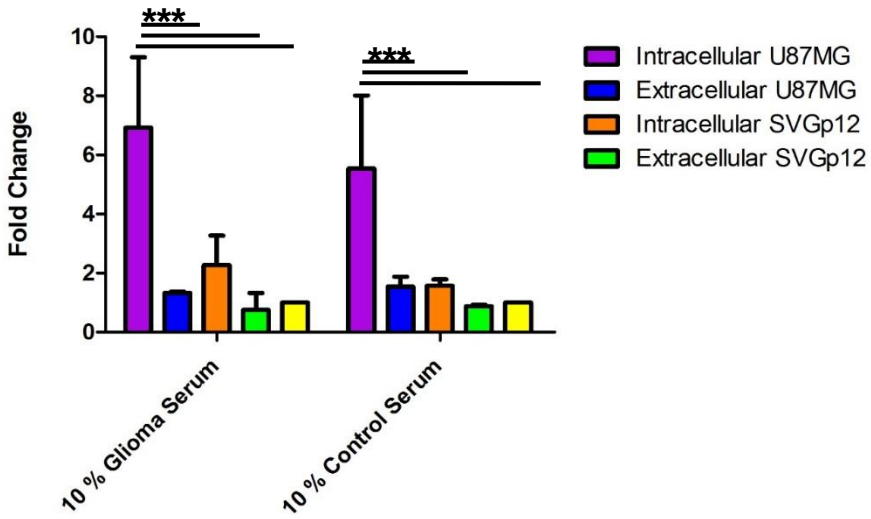


Figure 5.2 Comparison of miR-34a expression in U87MG and SVGp12 cell lines following serum replacement with 60+ patient serum pools. MiR-34a expression in U87MG cells cultured in 10 % 60+ GBM patient serum was significantly higher intracellularly compared to extracellular expression. Intracellular U87MG expression in 60+ GBM serum was significantly higher compared to intracellular SVGp12 or U87MG cells cultured in 10 % FBS. The same trend was observed in cells cultured in 10 % 60+ control patient serum. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by two-way ANOVA with Tukey *post-hoc* test. Yellow bar represents miR-34a expression in U87MG and SVGp12 cells cultured in 10 % FBS set to a standard value of 1 from which the fold change of all other bars were calculated, all serum weaning data were normalised to miR-34a expression in the corresponding serum pool. Significance indicated between groups by a solid black line, ***p < 0.001.

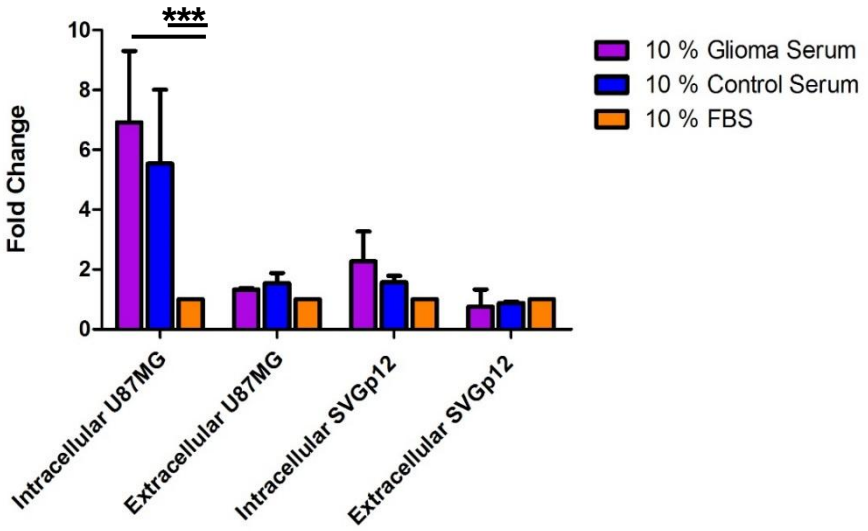


Figure 5.3 Comparison of miR-34a expression of cells cultured in 10 % 60+ GBM patient and 10 % 60+ control patient serum pools. Intracellular miR-34a expression in U87MG cells was significantly higher when cultured in both 60+ patient pools compared to those cultured in 10 % FBS. No significant difference in intracellular U87MG miR-34a expression was observed between cells cultured in 10 % GBM patient serum and those cultured in 10 % control patient serum. No significant difference in expression was observed both intracellularly and extracellularly between the three serum conditions for SVGp12 cells. Data shown as mean of replicates (n = 3 per group), plus SD, analysed by two-way ANOVA with Tukey *post-hoc* test, all serum weaning data were normalised to miR-34a expression in the corresponding serum pool. Significance between groups indicated by a solid black line, ***p < 0.001.

5.2.1.3 Expression of MiR-92a in Male Patient Serum Pools compared to U87MG and SVGp12 Cells Cultured in 10 % FBS

Prior to serum replacement, the expression of miR-92a in the male patient serum pools to be used for weaning was compared to the expression of U87MG and SVGp12 cells cultured in 10 % FBS. Initial comparison of miR-92a expression in the two serum pools showed no significant difference in expression (p < 0.001) of miR-92a in the male glioblastoma serum pool compared to the male control serum pool (Figure 5.4). MiR-92a expression was significantly lower (p < 0.001) in both male serum pools compared to intracellular miR-92a expression in U87MG and SVGp12 cells, and spent media containing 10 % FBS (Figure 5.4). Extracellular miR-92a expression in the spent media of U87MG cells cultured in 10 % FBS was significantly higher (p < 0.001) compared to intracellular U87MG miR-92a expression (Figure 5.4). Extracellular miR-92a expression in the spent media of U87MG cells cultured in 10 % FBS was not significantly different (p > 0.001) when compared to extracellular miR-92a expression of SVGp12 cells cultured in 10 % FBS (Figure 5.4).

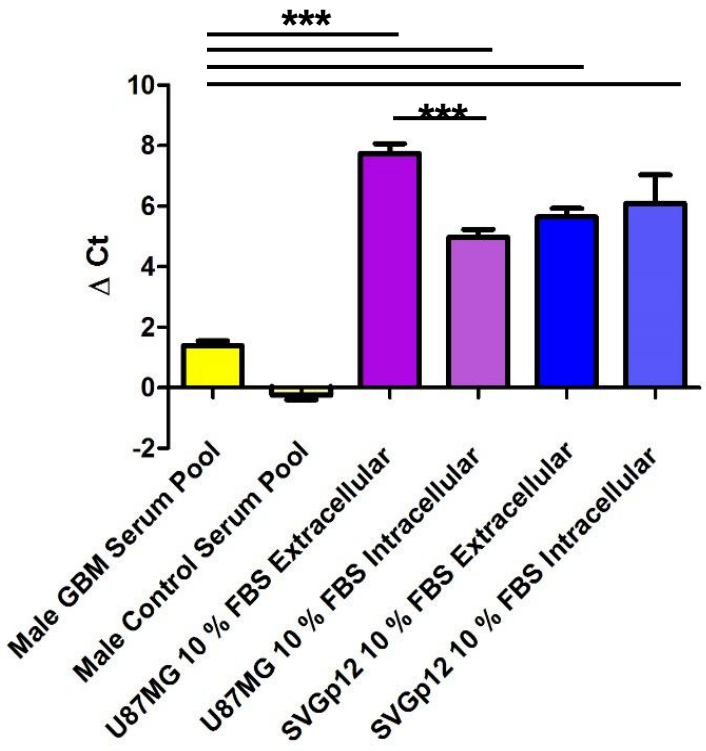


Figure 5.4 Comparison of miR-92a expression in male glioblastoma serum pool and male control patient serum pool. MiR-92a expression in both male patient serum pools was significantly lower compared to U87MG and SVGp12 miR-92a expression when cultured in 10 % FBS. Extracellular U87MG miR-92a expression was significantly higher compared to intracellular U87MG expression and extracellular SVGp12 expression. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by one-way ANOVA with Tukey *post-hoc* test. Significance indicated between groups by a solid black line, ***$p < 0.001$.

5.2.1.4 Expression of MiR-92a in U87MG and SVGp12 cells following Serum Replacement with Male Patient Serum Pools

After establishing miR-92a expression in the male glioblastoma patient serum pool and male control patient pool compared to intracellular expression in U87MG and SVGp12 cells and spent media containing 10 % FBS, cells were weaned onto the male patient pools and the change in miR-92a expression was determined compared to cells cultured in FBS. The culture of U87MG cells in both male glioblastoma serum and male control serum pools resulted in no significant change ($p > 0.001$) in miR-92a expression either intracellularly or extracellularly (Figure 5.5).

The culture of SVGp12 cells in the male glioblastoma serum pool resulted in a significant increase ($p < 0.001$) in intracellular miR-92a expression compared to extracellular miR-92a expression and SVGp12 cells cultured in FBS (Figure 5.5). The culture of SVGp12 cells in male control patient serum resulted no significant difference

of miR-92a intracellular expression ($p < 0.001$) compared to extracellular expression (Figure 5.5). When compared to SVGp12 cells cultured in FBS, intracellular and extracellular miR-92a expression of SVGp12 cells cultured in male control patient serum was not significantly different ($p > 0.001$, Figure 5.6).

Comparison of the effect of the two patient serum pools on U87MG and SVGp12 miR-92a expression showed no significant difference ($p > 0.001$) in miR-92a expression between U87MG cells cultured in male glioblastoma serum and those cultured male control serum (Figure 5.6). Intracellular miR-92a expression of SVGp12 cells was significantly higher ($p < 0.001$) in cells cultured in 10 % male glioblastoma patient serum compared to those cultured in 10 % control patient serum and 10 % FBS. Extracellular miR-92a expression in SVGp12 cells showed no significant difference ($p > 0.001$) in expression between the three serum conditions (Figure 5.6).

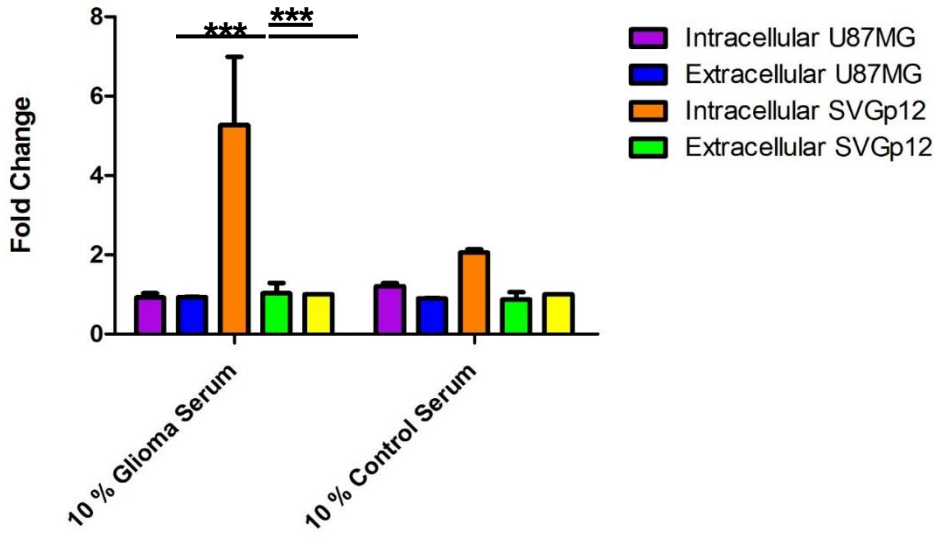


Figure 5.5 Comparison of miR-92a expression in U87MG and SVGp12 cells following culture in 10 % male patient serum pools. MiR-92a expression was significantly higher intracellularly in SVGp12 cells cultured in 10 % male GBM serum compared to extracellular SVGp12 and intracellular U87MG miR-92a expression. Yellow bar represents miR-92a expression in U87MG and SVGp12 cells cultured in 10 % FBS set to a standard value of 1 from which the fold change of all other bars were calculated, all serum weaning data were normalised to miR-92a expression in the corresponding serum pool. Significance indicated between groups by a solid black line, ***$p < 0.001$.

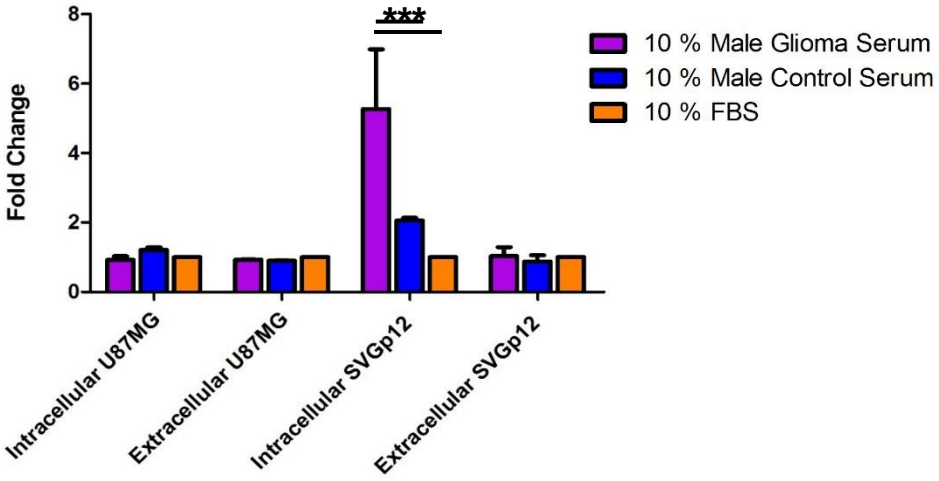


Figure 5.6 Expression of miR-92a in cell lines following serum replacement. U87MG miR-92a expression was not significantly different between the three serum conditions. Intracellular miR-92a expression in SVGp12 cells cultured in 10 % male GBM serum was significantly higher compared to cells cultured in 10 % male control serum and 10 % FBS. No significant difference was observed in intracellular miR-92a expression in SVGp12 cells cultured in 10 % male control patient serum and those cultured in 10 % FBS. Extracellular miR-92a expression in SVGp12 cells was not signficantly different between the three serum conditions. Data shown as mean of replicates (n = 3 per group), plus SD, analysed by two-way ANOVA with Bonferroni *post-hoc* test, all serum weaning data were normalised to miR-92a expression in the corresponding serum pool. Significance between groups indicated by a solid black line, ***$p < 0.001$.

5.2.1.5 Expression of MiR-20a in Glioblastoma Patient Serum compared to U87MG and SVGp12 Cells Cultured in 10 % FBS and following Serum Replacement

Prior to serum replacement, miR-20a expression in the glioblastoma patient serum pool and the control patient serum pool to be used for weaning was compared to miR-20a expression in U87MG and SVGp12 cells. MiR-20a expression was detected intracellularly in U87MG cells cultured in 10 % FBS only. MiR-20a expression was not significantly different (p > 0.001) in the control patient serum pool compared to intracellular U87MG miR-20a expression (Figure 5.7). MiR-20a expression in the glioblastoma patient serum pool exhibited no significant difference (p > 0.001) compared to miR-20a expression in U87MG cells cultured in 10 % FBS (Figure 5.7).

The culture of U87MG cells in both glioblastoma patient serum and control patient serum showed no significant difference (p > 0.001) in intracellular miR-20a expression compared to U87MG cells cultured in FBS (Figure 5.8).

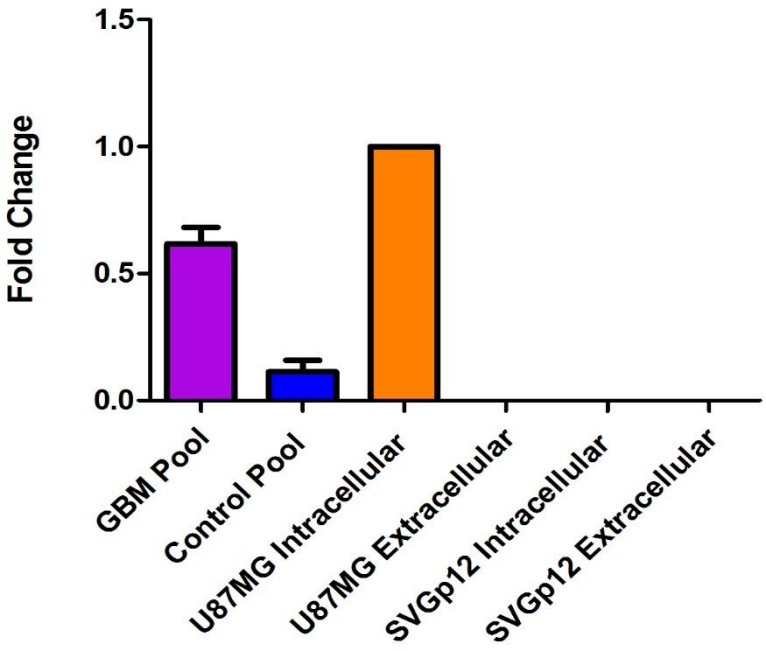


Figure 5.7 MiR-20a expression in glioblastoma patient pooled serum and control patient pooled serum compared to intracellular U87MG miR-20a expression. MiR-20a expression was not significantly different in the control patient serum pool compared to intracellular U87MG expression. MiR-20a expression in the glioblastoma patient serum pool exhibited no significant difference in expression compared to U87MG cells or control patient serum pool. Data shown as mean of replicates (n = 3 per group) plus SD analysed by Kruskal-Wallis one way ANOVA with Dunn's *post-hoc* test. Significance indicated between groups by a solid black line, p > 0.05.

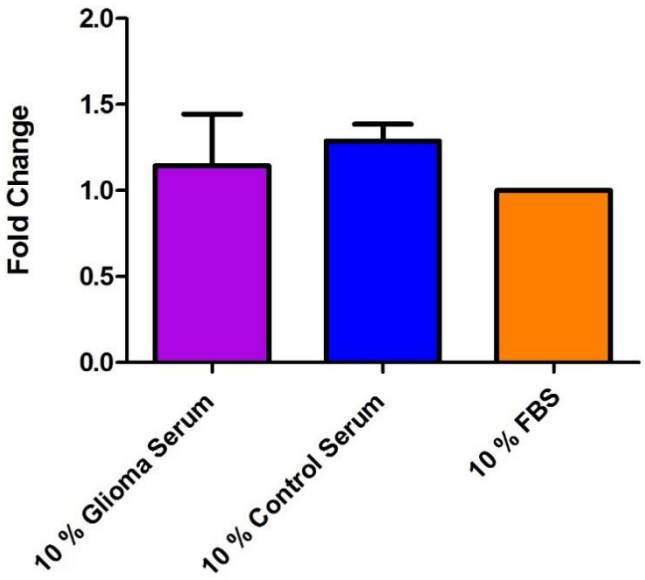


Figure 5.8 Intracellular miR-20a expression in U87MG cells following culture in 10 % GBM patient and control patient serum pools. Intracellular miR-20a expression was not significantly different between U87MG cells cultured in glioblastoma patient serum or control patient serum or compared to those cultured in FBS. Data shown as mean of replicates (n = 3 per group) plus SD, all serum weaning data were normalised to miR-20a expression in the corresponding serum pool. Data were analysed by Kruskal-Wallis one-way ANOVA with Dunn's *post-hoc* test, $p > 0.05$.

5.2.1.7 Expression of MiR-30c in 20-39 Glioblastoma Patient Serum compared to U87MG and SVGp12 Cells Cultured in 10 % FBS and following Serum Replacement

Prior to serum replacement, miR-30c expression in the 20-39 glioblastoma patient serum pool and the 20-39 control patient serum pool to be used for weaning was compared to miR-30c expression in U87MG and SVGp12 cells. MiR-30c expression was detected intracellularly in U87MG cells cultured in 10 % FBS only. MiR-30c expression was not significantly different ($p > 0.001$) in the 20-39 glioblastoma patient serum pool compared to U87MG expression (Figure 5.9). Expression of miR-30c in the 20-39 control patient serum pool was not significantly different ($p > 0.001$) to miR-30c expression in U87MG cells (Figure 5.9). Intracellular expression of U87MG cells cultured in 20-39 glioblastoma patient serum exhibited a higher expression of miR-30c expression compared to those cultured in FBS (Figure 5.10). U87MG cells cultured in 20-39 control patient serum exhibited no significant change ($p > 0.01$) in expression compared to those cultured in FBS (Figure 5.10).

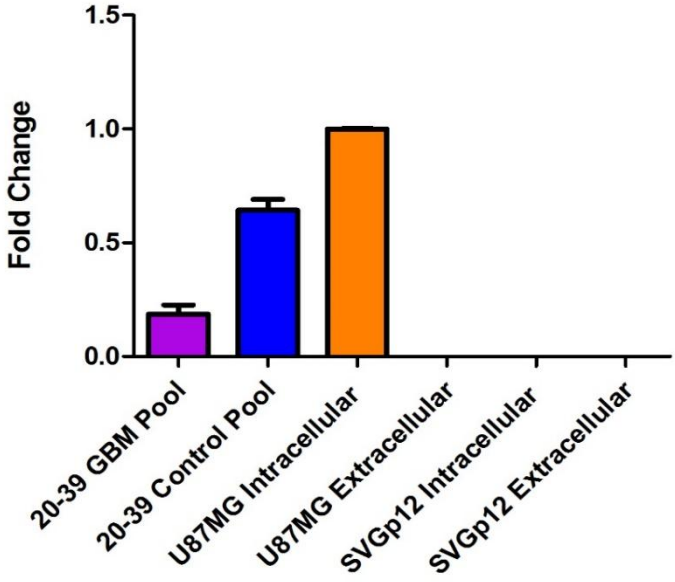


Figure 5.9 MiR-30c expression in 20-39 glioblastoma patient pooled serum and 20-39 control patient pooled serum compared to intracellular U87MG miR-30c expression. MiR-30c expression was not significantly different in the 20-39 glioblastoma patient serum pool compared to 20-39 control pool expression and intracellular U87MG cultured in 10 % FBS. MiR-30c expression in the 20-39 control patient serum pool exhibited no significant difference in expression compared to U87MG cells cultured in 10 % FBS. Data shown as mean of replicates (n = 3 per group) plus SD analysed by Kruskal-Wallis one way ANOVA with Dunn's *post-hoc* test, $p > 0.001$.

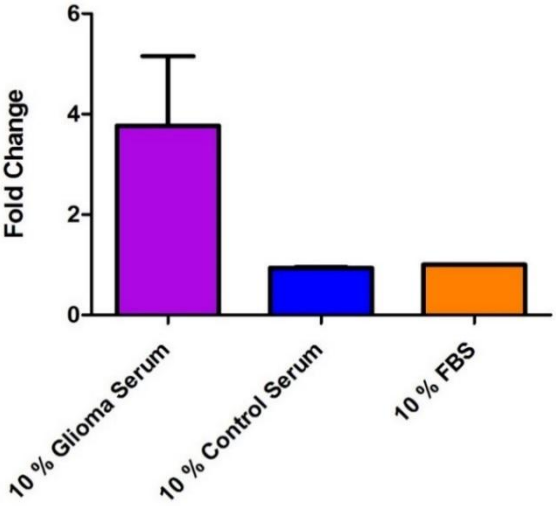


Figure 5.10 Intracellular miR-30c expression in U87MG cells following culture in 10 % 20-39 patient serum pools. Intracellular miR-30c expression was not significantly different in U87MG cells cultured in 20-39 glioblastoma patient serum compared to those cultured in 10 % 20-39 control patient serum and 10 % FBS. MiR-30c expression was not significantly different in U87MG cells cultured in 20-39 control patient serum compared to those cultured in 10 % FBS. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by Kruskal-Wallis one-way ANOVA with Dunns *post-hoc* test, all serum weaning data were normalised to miR-30c expression in the corresponding serum pool, $p > 0.01$.

5.2.2 MicroRNA Expression in Glioblastoma Tissue

Data from Section 5.2.1 demonstrated that some of the miRNAs within the biomarker panel may originate from the tissue itself due to the high intracellular expression of these miRNAs *in vitro.* To further determine whether miRNAs present within the circulation originate from the glioblastoma itself, the expression of miRNAs was measured in tumour tissue samples. As the incidence of glioblastoma is most prevalent in individuals over the age of 60 years and three of the four biomarkers validated in Section 4.2.5 are all present in the serum of glioblastoma patients over the age of 60 years, tissue samples were obtained from patients of this age group. Age-matched tissue samples from one male and one female patient were obtained and analysed separately to determine whether there were any gender differences in tissue miRNA expression as observed in serum.

Analysis of the 84 miRNAs in the brain cancer panel identified 28 miRNAs that were highly expressed in the male tissue (Figure 5.11A). MiR-124a showed the highest level of expression in male tissue with a 6.04 fold increase, and 17-5p, 181b-5p, 182-5p, 183-5p and 184 exhibited more than a five-fold change in expression. MiR-146b-5p and 9-5p had a greater than four-fold change in expression and miR-19b-3p, 203a, 30b-5p, 30c-5p and 7-5p had a greater than three-fold change in expression. The remaining miRNAs exhibited at least a two-fold increase in expression in the male tissue. Ten miRNAs showed reduced expression in male glioblastoma tissue (Figure 5.11B), miR-23a-3p showed the greatest decrease in expression of 0.06 fold, the next two miRNAs with the greatest decrease in expression were miR-15b-5p and miR-21-5p with a 0.17 and 0.18 fold decrease, respectively. Three of the five miRNAs identified in Section 4.2.4 were expressed in male tissue, miR-30c, miR-92a and miR-150. Expression of miR-92a showed a 2.12 fold increase in expression in the tissue of the male glioblastoma patient compared to age matched control tissue and miR-30c and miR-150 showed a 3.14 fold and 2.00 fold increase in expression, respectively (Figure 5.13A).

Five miRNAs showed a high level of expression in the female glioblastoma tissue (Figure 5.12A), all five miRNAs exhibited a greater than two-fold increase in expression with miR-184 having the greatest increase in expression. 25 miRNAs showed reduced

expression in female glioblastoma tissue with miR-130a-3p having the greatest decrease in expression of 0.06 fold (Figure 5.12B). Two of the four validated serum biomarkers, miR-92a and miR-30c, were detected in the female tissue. MiR-92a and miR-30c were both observed to have reduced expression in the female tissue by a fold change of 0.10 and 0.14 respectively (Figure 5.12B). MiRNAs 185-5p and 184 were up-regulated in both male and female samples, both male and female showed a similar fold increase of 2.39 and 2.36 fold for miR-185-5p, however miR-184 had a greater increase in expression of 5.76 fold in the male sample compared to 2.50 in the female sample. MiR-130a-3p, 21-5p and 23a-3p were all down-regulated in both male and female samples. MiR-130a-3p had a greater decrease in fold change in the female sample of 0.06 fold compared to 0.26 fold whereas 21-5p and 23a-3p had a greater decrease in expression in the male sample of 0.18 and 0.06 compared to the female sample expression of 0.40 and 0.28 fold.

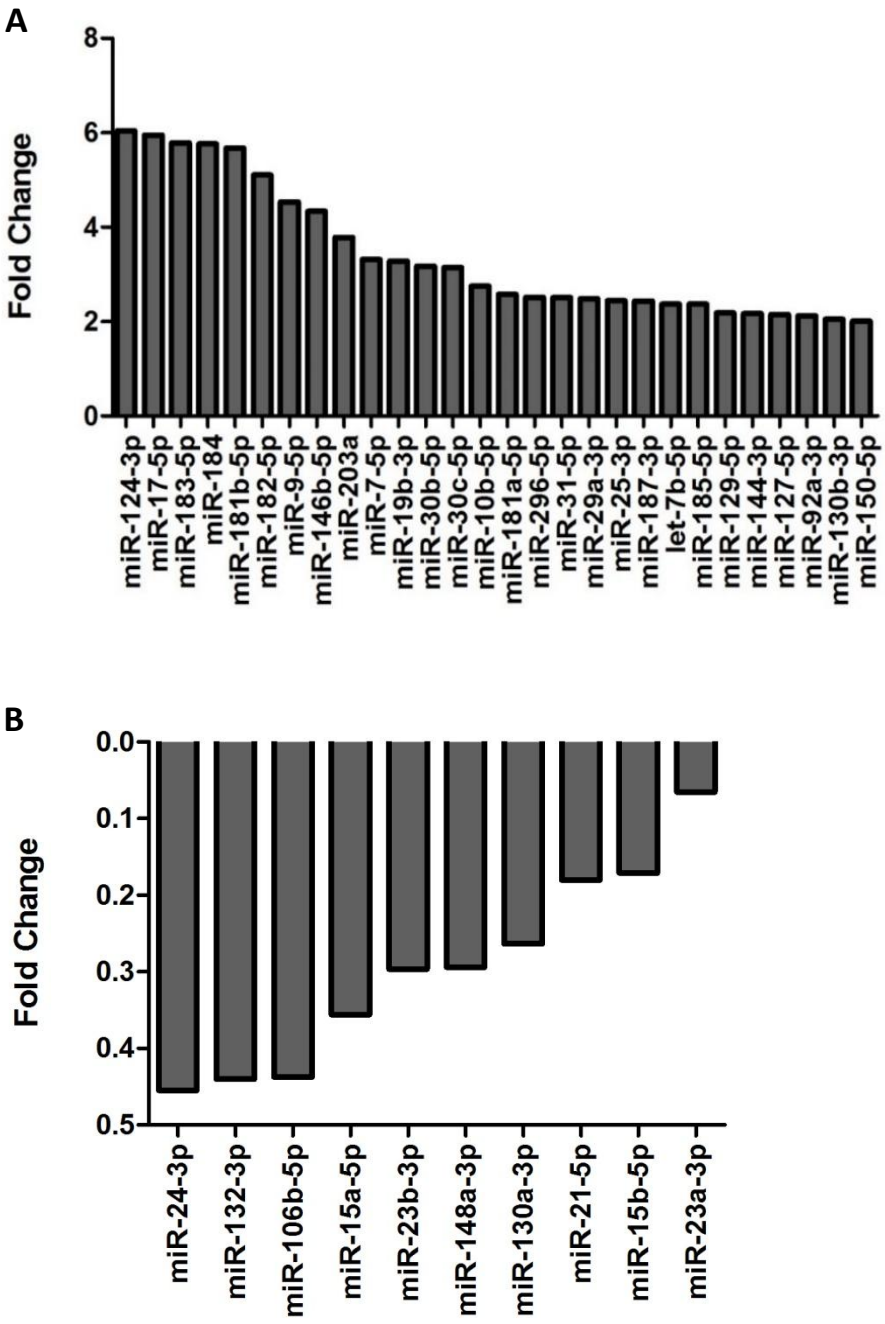


Figure 5.11 Fold Change of miRNAs in 60+ male glioblastoma patient tissue. A) 28 miRNAs exhibited an increase in expression in glioblastoma tissue obtained from a patient aged over 60 years (n=1), miR-124-3p showed the highest expression compared to matched control tissue. B) 10 miRNAs exhibited reduced expression in glioblastoma tissue obtained from a male patient aged over 60 years (n=1), miR-23a-3p showed the greatest decrease in expression compared to matched control tissue.

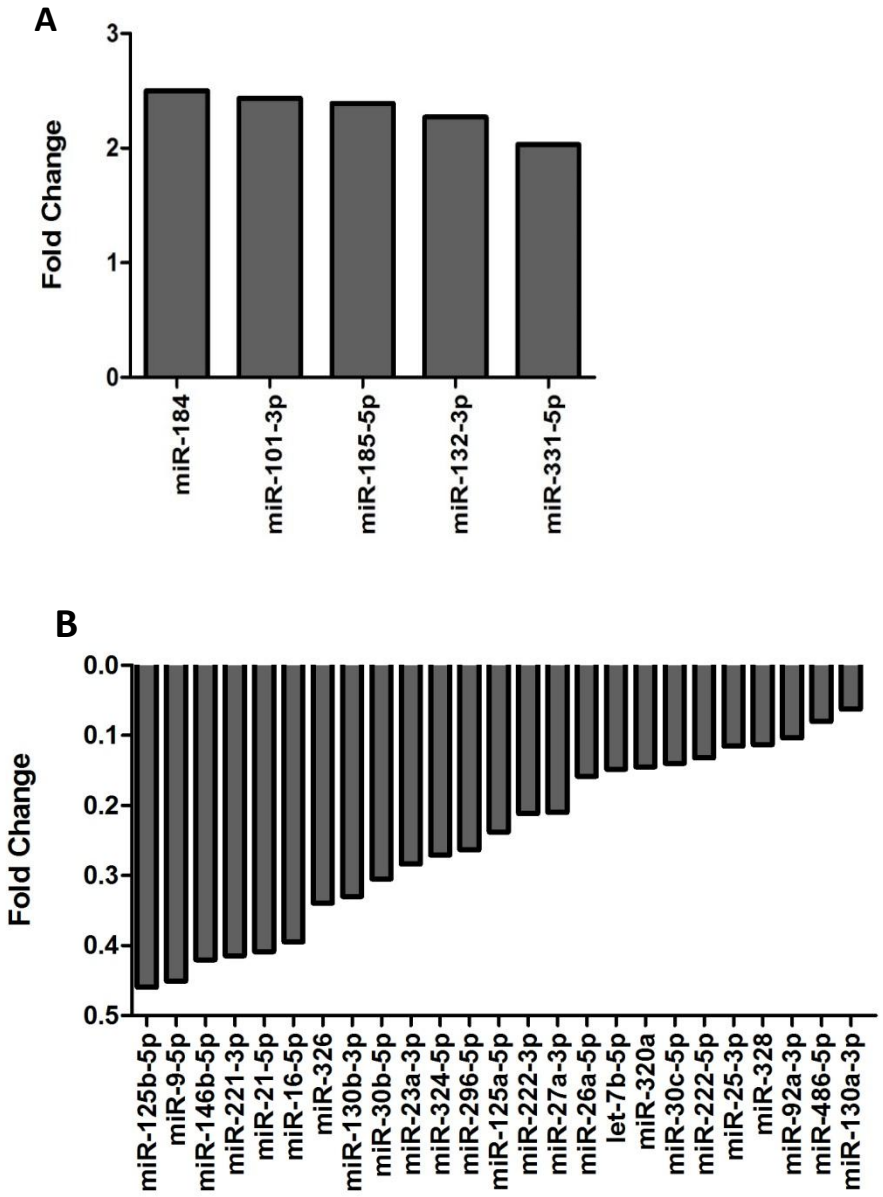


Figure 5.12 Fold Change of miRNAs in 60+ female glioblastoma patient tissue. A) Four miRNAs showed an increased expression in the tissue of a female glioblastoma patient aged over 60 years (n=1), miR-184 showed the greatest increase in expression compared to matched control tissue. B) 25 miRNAs exhibited reduced expression in the tissue of a female glioblastoma patient aged over 60 years (n=1). MiR-130a-3p showed the greatest decrease in expression compared to matched control tissue.

5.2.3 MicroRNA Expression in TCGA

Following identification of four miRNAs with significant altered expression in the sera of glioblastoma patients, microarray data obtained from the TCGA was analysed by Josie Hayes from the Leeds Institute of Molecular Medicine to identify whether the expression of the miRNA panel correlated with miRNA expression in tissue in a larger cohort of 558 patients which could be grouped by age and gender. The microarray data was analysed to determine the expression of the miRNAs in tissue, age and gender differences, and prognostic potential. As it has not been previously shown to have a role in glioblastoma, tissue expression of miR-150 was also investigated to determine whether this miRNA is dysregulated in glioblastoma.

MiR-34a expression was significantly higher ($p < 0.0001$) in the tissue of glioblastoma patients (Table 5.1) and expression was correlated with age ($r = 0.23$, $p < 0.0001$), with older patients exhibiting higher expression (Figure 5.15A). Higher expression of miR-34a was associated with a poorer prognosis ($p < 0.05$) compared to individuals with a low expression (Figure 5.15B). Patients with a higher expression of miR-34a had a median survival time of 13.3 months and patients with a low expression having a median survival time of 13.9 months. Analysis of miR-34a expression based on gender showed no significant difference ($p >0.05$) in miR-34a expression between male and female groups (Figure 5.15C).

MiR-92a was significantly up-regulated ($p < 0.0001$) in the tissue of glioblastoma patients compared to non-cancerous patients (Table 5.1). Expression was not correlated with age ($r = -0.15$, $p < 0.0001$), (Figure 5.16A). Patients with a higher expression of tissue miR-92a did not exhibit a significant difference ($p > 0.05$) in median survival time compared to those without (Figure 5.16B). Patients with high expression of miR-92a had a median survival time of 14.4 months and patients with low expression of miR-92a had a median survival of 12.9 months. Analysis of miR-92a based on gender exhibited no significant difference ($p > 0.05$) in expression of miR-92a in tissue between male and female patients (Figure 5.16C).

MiR-20a was significantly up-regulated ($p < 0.0001$) in the tissue of glioblastoma patients (Table 5.1), furthermore, expression was inversely correlated with age ($r = -0.15$, $p < 0.0001$) with older patients exhibiting a lower expression of miR-20a (Figure

5.17A). Higher expression of miR-20a in the tissue of glioblastoma patients was associated with a better prognosis (Figure 5.17B). Patients with a higher expression of miR-20a had a median survival of 15.3 months and patients with a low expression of miR-20a had a median survival of 12.6 months. Analysis of miR-20a expression grouped by gender showed no significant difference ($p > 0.05$) in expression between male and female groups (Figure 5.17C).

MiR-20a and 92a are located within the genome in a cluster of miRNAs which are transcribed together therefore expression of these two miRNAs in each individual patient was analysed to determine whether expression was correlated. Analysis of patient miR-92a and miR-20a expression showed a high correlation ($r = 0.46$, $p < 0.0001$) in expression for these two miRNAs (Figure 5.17D).

Analysis of miR-30c and miR-150 in patient tissue found no significant difference ($p > 0.05$) in expression between glioblastoma and control patients (Table 5.1). Furthermore, no difference in expression was observed between age groups for miR-30c and miR-150 ($r = -0.02$ and -0.03 respectively, $p > 0.05$) or genders (Figures 5.18A and B and 5.19A and B). Survival analysis of miR-30c and miR-150 also showed no association with prognosis ($p > 0.05$).

Table 5.1 Log fold change expression of serum miRNA biomarkers in glioblastoma tissue obtained from TCGA analysed by Josie Hayes.

MiRNA	Log fold Change		p value
miR-34a-5p	1.30	Increased	5.3×10^{-4}
miR-92a-3p	1.21	Increased	5.6×10^{-9}
miR-20a-5p	1.37	Increased	6.3×10^{-7}
miR-30c-5p	0.301	No change	0.24
miR-150-5p	0.031	No change	0.87

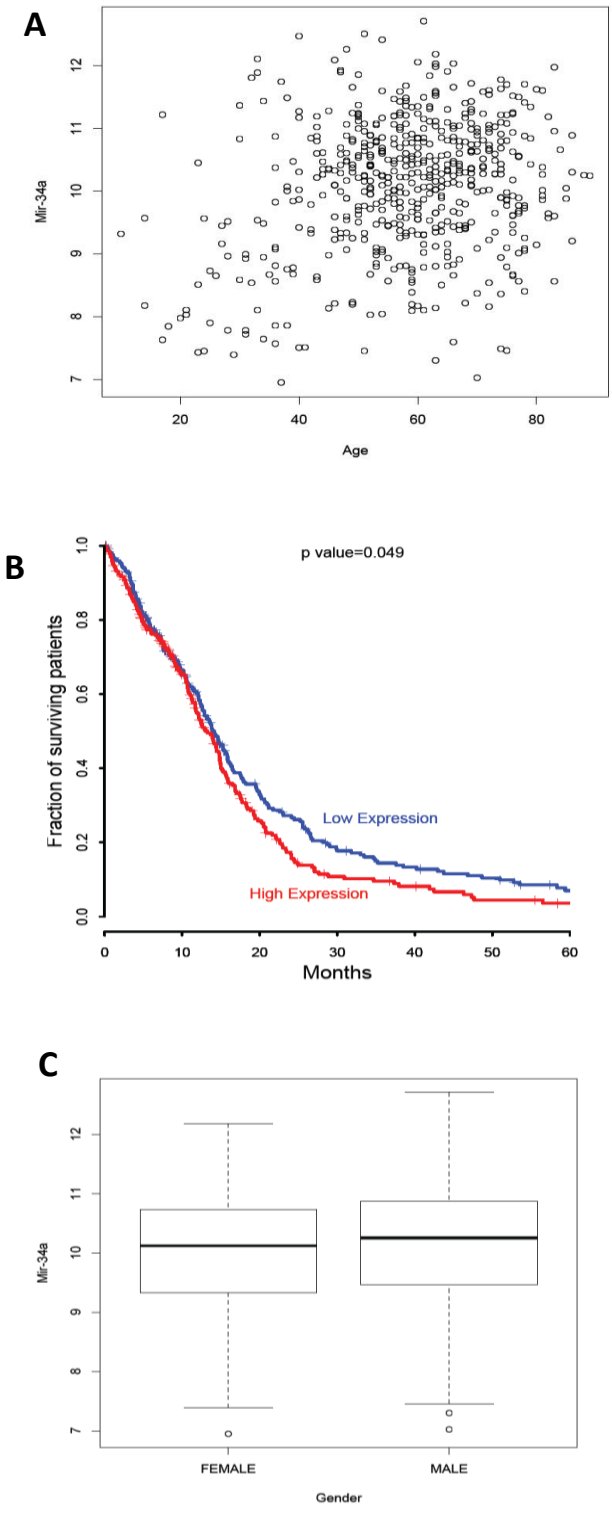


Figure 5.13 Analysis of miR-34a expression in glioblastoma tissue using the TCGA dataset. A) Expression of miR-34a in glioblastoma tissue was correlated with age determined by Pearson's correlation $r = 0.23$ ****$p < 0.0001$. B) Patients with a low expression of miR-34a exhibited a better prognosis than those with an up-regulation determined by log-rank above and below the median *$p < 0.05$. C) No gender difference in tissue miR-34a expression was observed, $p > 0.05$. High and low miRNA expression was determined as above and below the median. Analysis and figures produced by Josie Hayes (Leeds Institute of Molecular Medicine).

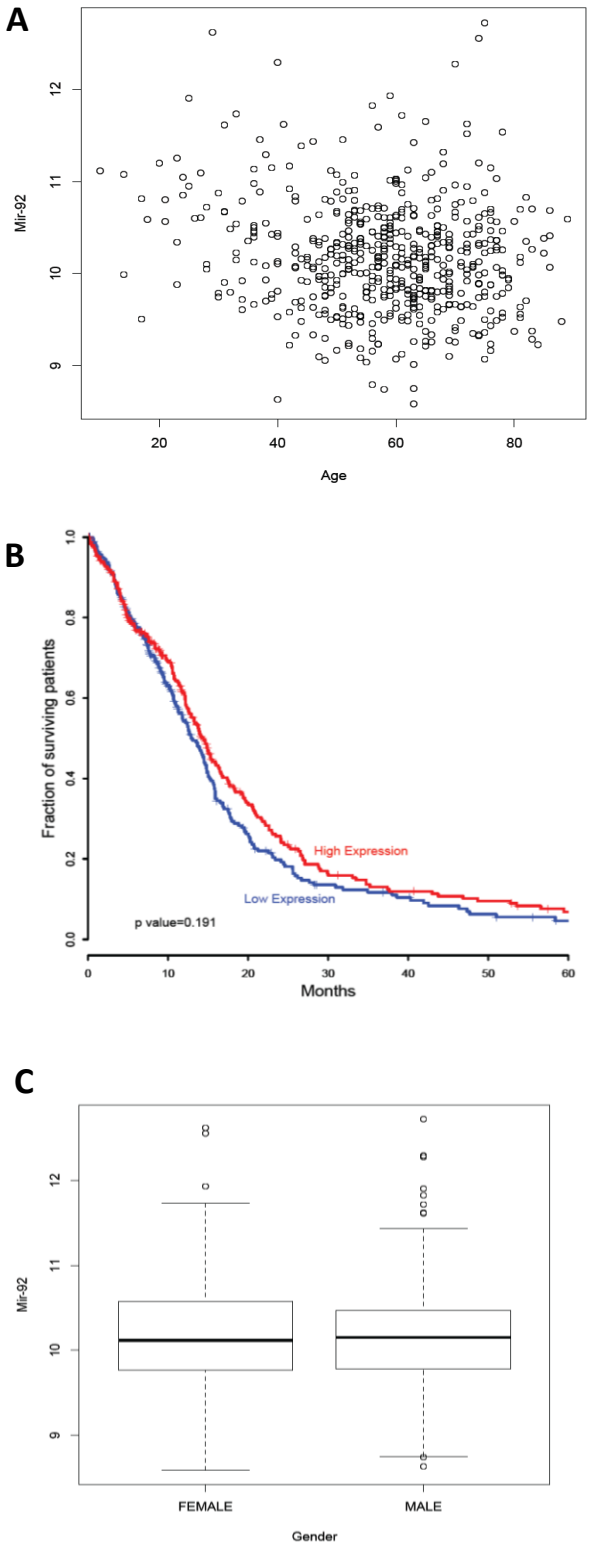


Figure 5.14 Analysis of miR-92a expression in glioblastoma patient tissue using the TCGA dataset. A) Expression of tissue miR-92a was not correlated with age determined by Pearson's correlation. r = -0.15, $p < 0.0001$. B) Patients with a higher expression of miR-92a did not exhibit a significantly better prognosis than those with a low expression by log-rank above and below the median, $p > 0.05$. C) No gender difference in tissue miR-92a expression was observed, $p > 0.05$. High and low miRNA expression was determined as above and below the median. Analysis and figures produced by Josie Hayes (Leeds Institute of Molecular Medicine).

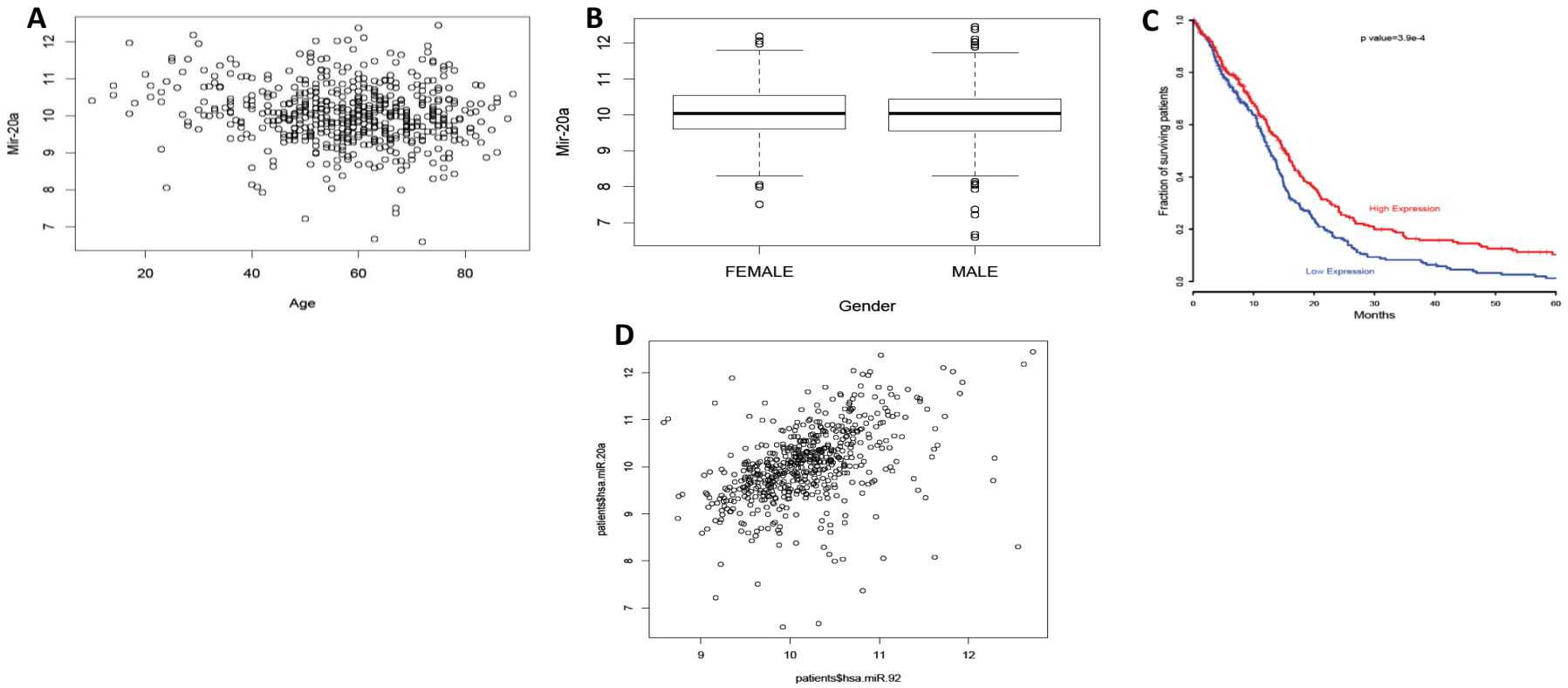


Figure 5.15 Analysis of tissue miR-20a expression using the TCGA dataset. A) Expression of tissue miR-20a was not correlated with age determined by Pearson's correlation. r = -0.15, ****p < 0.0001 B) No significant difference in tissue miR-20a expression was observed between genders p > 0.05. C) Patients with a higher expression of miR-20a exhibited a better prognosis compared to those with a low expression determined by log-rank above and below the median, ****p < 0.0001 D) Expression of miR-92a and miR-20a was correlated in tissue determined by Pearson's correlation. r = 0.46, ****p < 0.0001. High and low miRNA expression was determined as above and below the median. Analysis and figures produced by Josie Hayes (Leeds Institute of Molecular Medicine).

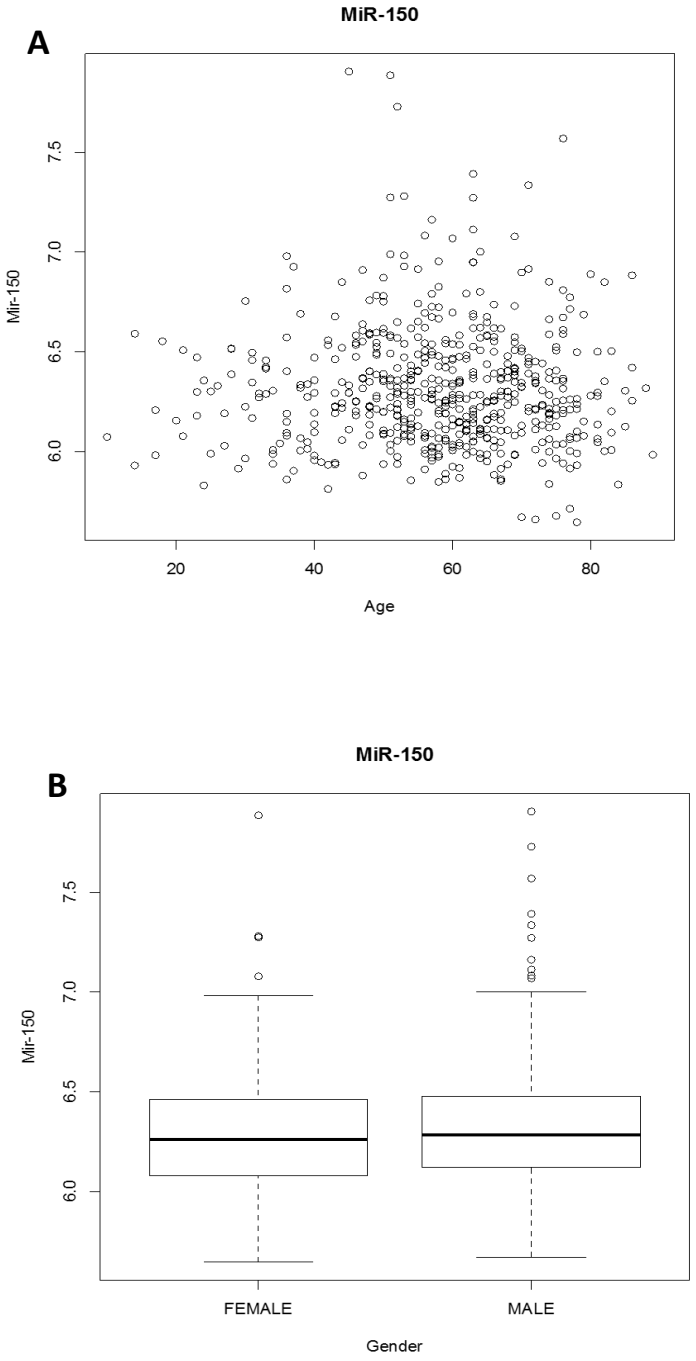


Figure 5.16 Analysis miR-150 expression in glioblastoma patient tissue using the TCGA dataset. A) Expression of tissue miR-150 was not correlated with age of glioblastoma patients determined by Pearson's correlation. r = -0.03, p > 0.05. B) No significant difference in miR-150 was observed between genders, p > 0.05. Analysis and figures produced by Josie Hayes (Leeds Institute of Molecular Medicine).

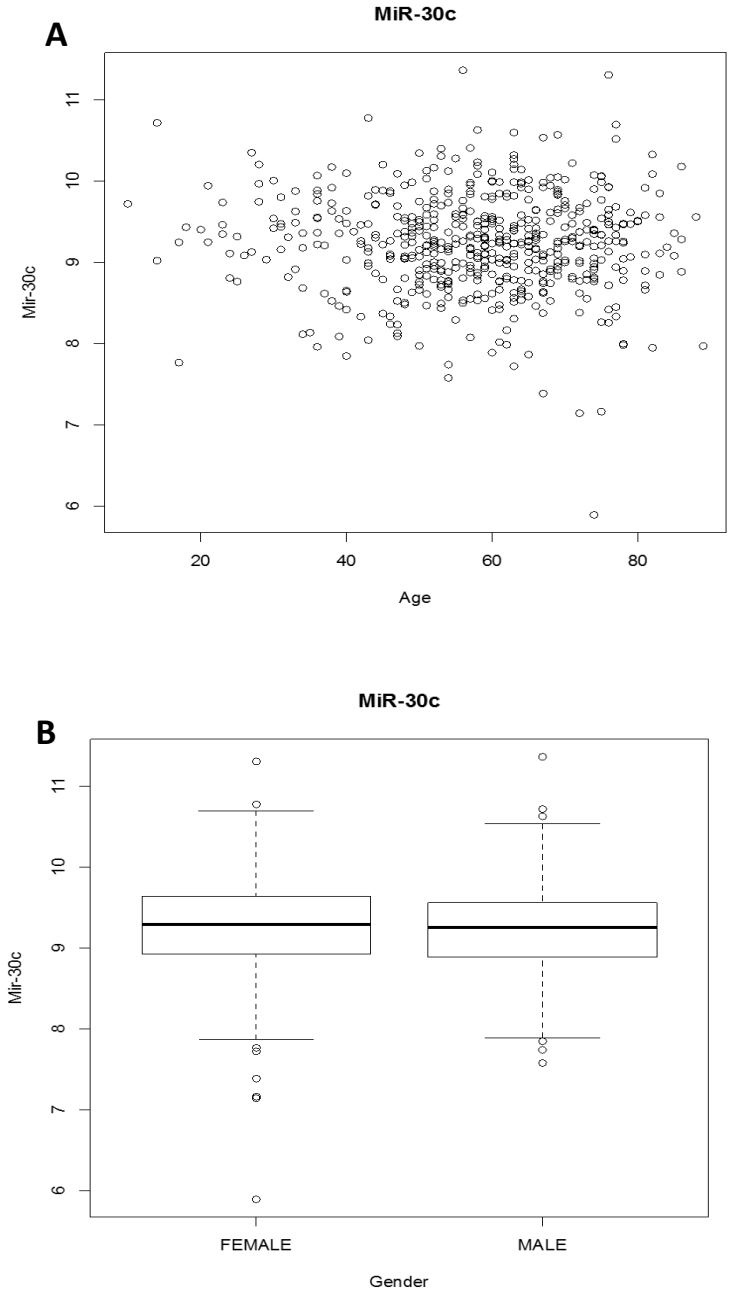


Figure 5.17 Analysis of miR-30c expression in glioblastoma patient tissue using the TCGA dataset. A) MiR-30c expression was not significantly correlated with age determined by Pearson's correlation. r = -0.02, p > 0.05. B) Expression of miR-30c was not significantly different between genders, p > 0.05. Analysis and figures produced by Josie Hayes (Leeds Institute of Molecular Medicine).

5.2.4 Expression of Serum Biomarkers in Matched Tissue

Following miScript analysis of miRNA expression in tissue and analysis of the four miRNA biomarkers tissue expression in the TCGA dataset, further studies were performed to determine the expression of the five biomarkers in patient tissue compared to matched serum samples. Matched tissue samples were not available for the 20-39 patient group therefore correlation between serum and tissue expression of miR-30c was not performed.

MiR-34a was found to be up-regulated in the serum of glioblastoma patients over the age of 60 years in Sections 4.2.4 and 4.2.5. MiR-34a is a known tumour suppressor and is down-regulated in glioblastoma tissue (Okada et al., 2014). The expression of miR-34a was determined in matched tissue and serum samples obtained from four patients, to determine the difference in expression between the sample types in order to elucidate whether patients used in this study also demonstrated reduced expression of miR-34a in the tissue as reported in previous studies and an up-regulation of miR-34a in the serum as observed in Sections 4.2.4 and 4.2.5, or alternatively whether miR-34a is similarly expressed in the tissue as observed in the TCGA data in Section 5.2.3. MiR-34a expression was higher in the serum of four glioblastoma patients over the age of 60 years compared to expression within the tissue obtained from the same patient (Figure 5.18).

In Sections 4.2.4 and 4.2.5 miR-92a expression was higher in the serum of male glioblastoma patients. Analysis of miR-92a expression in three matched serum and tissue samples showed a significantly lower expression of miR-92a in the serum of male glioblastoma patients compared to the expression of miR-92a in matched tissue samples (Figure 5.19).

MiR-20a expression was higher in the serum of a sub-group of glioblastoma patients in Sections 4.2.4 and 4.2.5. Comparison of miR-20a expression in matched serum and tissue samples showed no significant difference in expression between the two sample types (Figure 5.20).

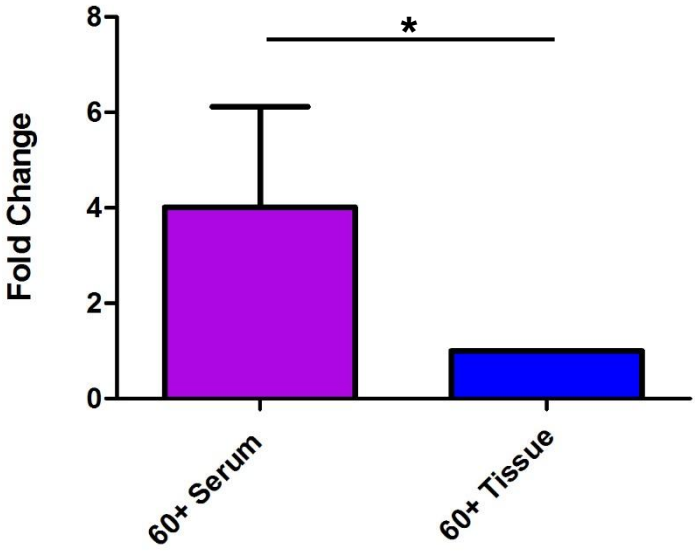


Figure 5.18 Expression of miR-34a in matched serum and tissue samples. Expression of miR-34a was higher in the serum of glioblastoma patients over the age of 60 years compared to matched tissue samples obtained from the same patient. Data shown as mean of four samples with SD, with the 60+ control tissue set at a standard value of 1, data analysed by Mann-Whitney U test, p = < 0.05.

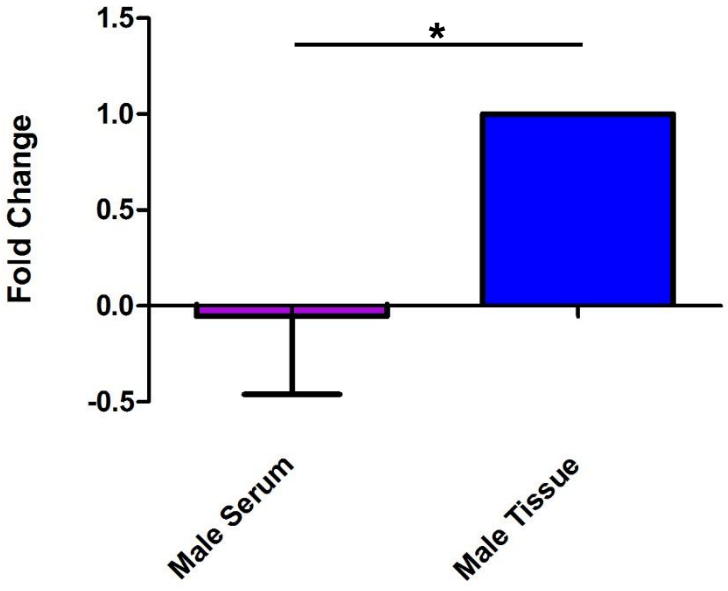


Figure 5.19 Expression of miR-92a in matched male glioblastoma patient serum and tissue samples. Expression of miR-92a was significantly lower in the serum of male glioblastoma patients compared to expression in matched tissue samples. Data shown as mean of triplicate samples plus SD, analysed by Mann-Whitney U test, p < 0.05.

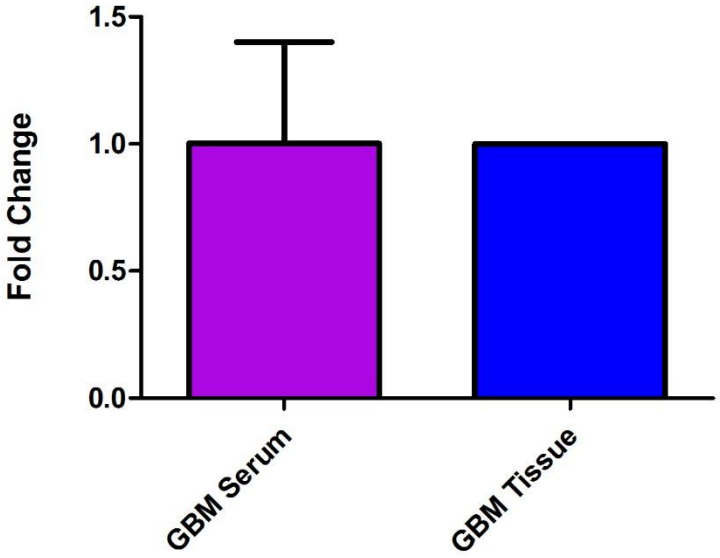


Figure 5.20 Comparison of miR-20a expression in matched serum and tissue samples from glioblastoma patients. Analysis of miR-20a expression in matched samples showed no significant difference in expression between serum and tissue samples. Data shown as mean of 5 samples plus SD, analysed by Mann-Whitney U test, $p > 0.05$.

5.2.5 *In Situ* Hybridisation of miRNA Biomarker Probes

Glioblastoma is composed of a group of heterogenous cells, differing in miRNA expression (Singh et al., 2012). To determine the localisation and population of cells within glioblastoma which may express the miRNA biomarkers identified within the serum and to determine any correlation in tissue expression between the TCGA and qPCR analysis in previous sections, *in situ* hybridization (ISH) of tissue sections was performed. Furthermore, the invasive nature of glioblastoma and indistinct tumour margins allows for the identification of miRNA expression in neighbouring non-cancerous cells that may be present on the sections. Identification of the localisation of miR-34a expression in particular could help to determine whether the relative high expression of this miRNA in the serum is due to secretion by neighbouring non-cancerous cells as an anti-proliferative signal. Due to the sensitive nature of the ISH protocol and optimisation required, three miRNA biomarkers were selected from the panel for investigation, miR-20a, miR-34a and miR-92a. MiR-30c was not included based on the non-significant tissue expression in the TCGA data in Section 5.2.2.

Tissue sections were selected from patients whose serum samples were used in Section 4.2.4 and miRNA expression of matched tissue samples was determined using qRT-PCR and fold change compared to serum biomarker expression for comparison with data obtained from ISH. Tissue sections were obtained from 12 patients, two male and female patients for each age group and tissue lysates were obtained for five of the patients for qPCR analysis. Optimisation of the ISH protocol was performed using the U6 small nucleolar RNA (snRNA), an endogenously expressed RNA, and miR-21, a miRNA with high expression in glioblastoma (Figure 5.21). U6 snRNA and miR-21 probes were incubated overnight at both 4 °C and 37 °C, to determine the optimum temperature for probe incubation. Positive results were observed for both positive control probes at 4 ° C and 37 °C. Following these results a final temperature of 37 °C was selected for ISH analysis of target miRNA. A scrambled miRNA probe was also used during optimisation studies which showed negative results at both temperatures.

Following optimisation of probe temperature, incubation times for the anti-DIG AP antibody and NBT-BCIP substrate were optimised. Sections were incubated for one hour each with anti-DIG AP and NBT-BCIP, two hours anti-DIG AP and one hour NBT-BCIP and two hours each with anti-DIG AP and NBT-BCIP. Incubation of sections for one hour with anti-DIG AP and one with NBT-BCIP showed positive staining for the U6 positive control probe and negative staining for the scrambled probe however, the miR-34a, miR-92a and miR-20a probes produced weak signal (Figure 5.22). Incubation for two hours with anti-DIG AP and one hour NBT-BCIP produced signal with the U6 positive control probes and the target miRNA probes and was negative for the scrambled probe (Figure 5.23). Incubation for two hours with both anti-DIG AP and NBT-BCIP was performed to determine whether the signal strength of the miRNA targets could be improved however non-specific probe binding was observed (Figure 5.24).

Following optimisation of the ISH protocol, analysis of tissue sections selected from patients used in the serum studies in Chapter 4 were performed. All tissue sections showed positive signal for the U6 snRNA positive control (Figures 5.22 and 5.23) and negative signal with the scrambled probe (Figures 5.24 and 5.25). BTNW 1336, obtained from a female 60+ glioblastoma patient showed extensive necrosis in the tissue section characterised by the lack of nuclear fast red counter staining due to a

reduced number of nuclei and non-specific probe signal. Patient tissue sections probed with the scrambled negative control all showed no signal (Figures 5.24 and 5.25).

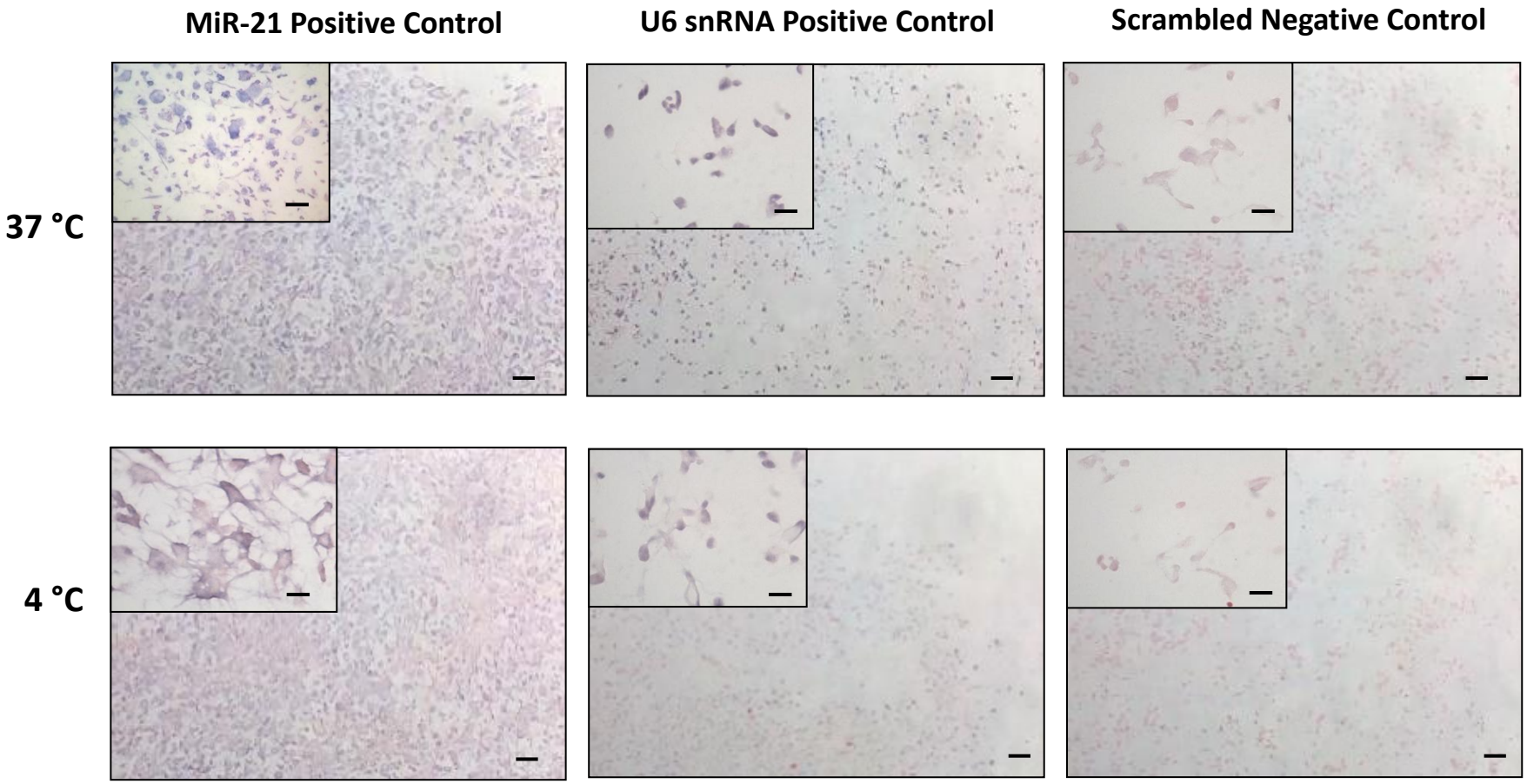


Figure 5.21 Optimisation of probe hybridisation temperature. Positive signals were obtained for both the miR-21 and U6 snRNA positive control probes and negative signals for the scrambled probe at both 4 ° C and 37 ° C. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

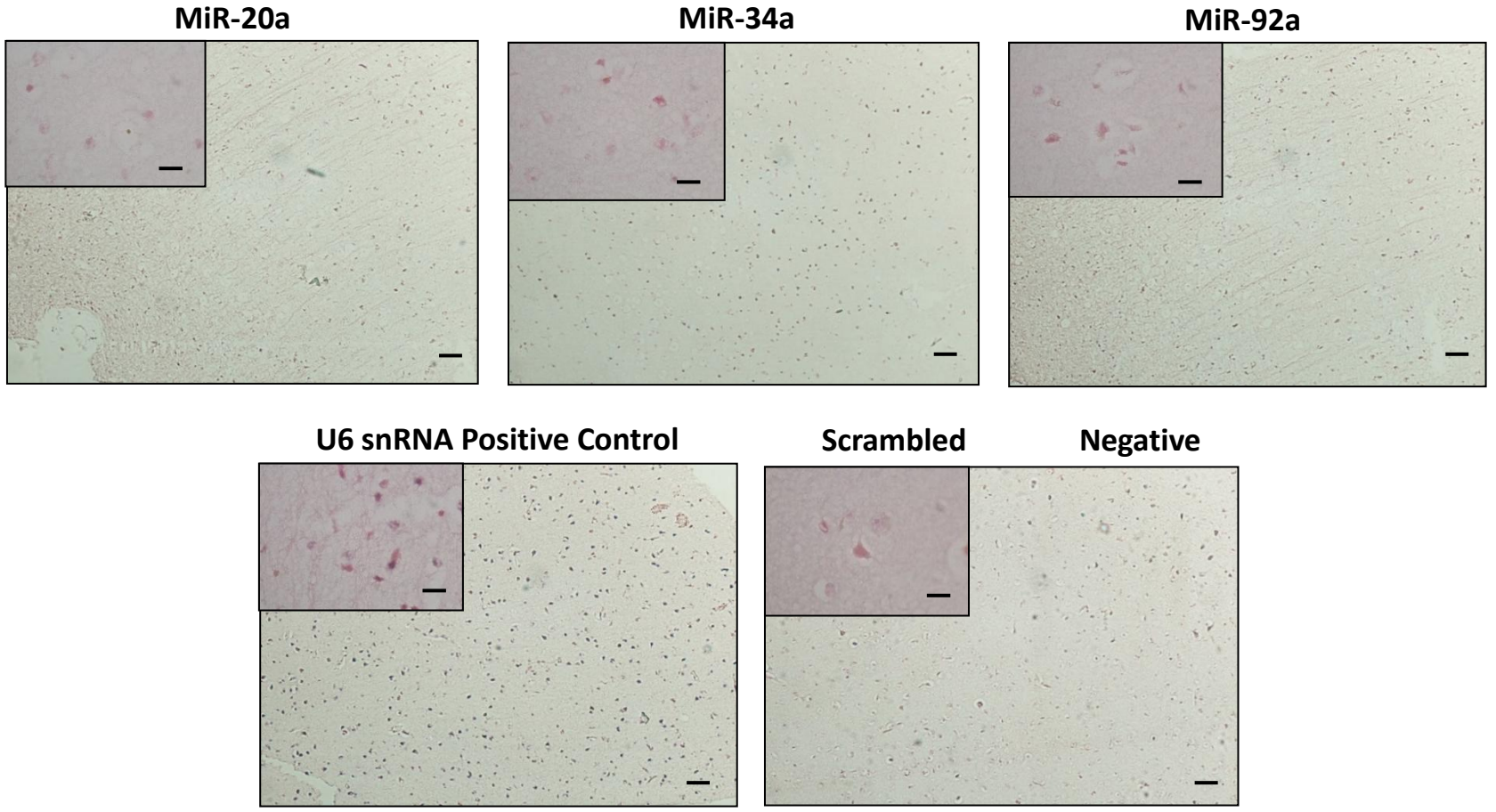


Figure 5.22 Optimisation of ISH Protocol - One hour antibody and NBT-BCIP incubation. Incubation of tissue sections with antibody for one hour at 37 ^{o}C followed by incubation with NBT-BCIP for one hour at 37 ^{o}C exhibited positive U6 signal and negative scrambled probe control and weak signal for the target miRNA probes. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

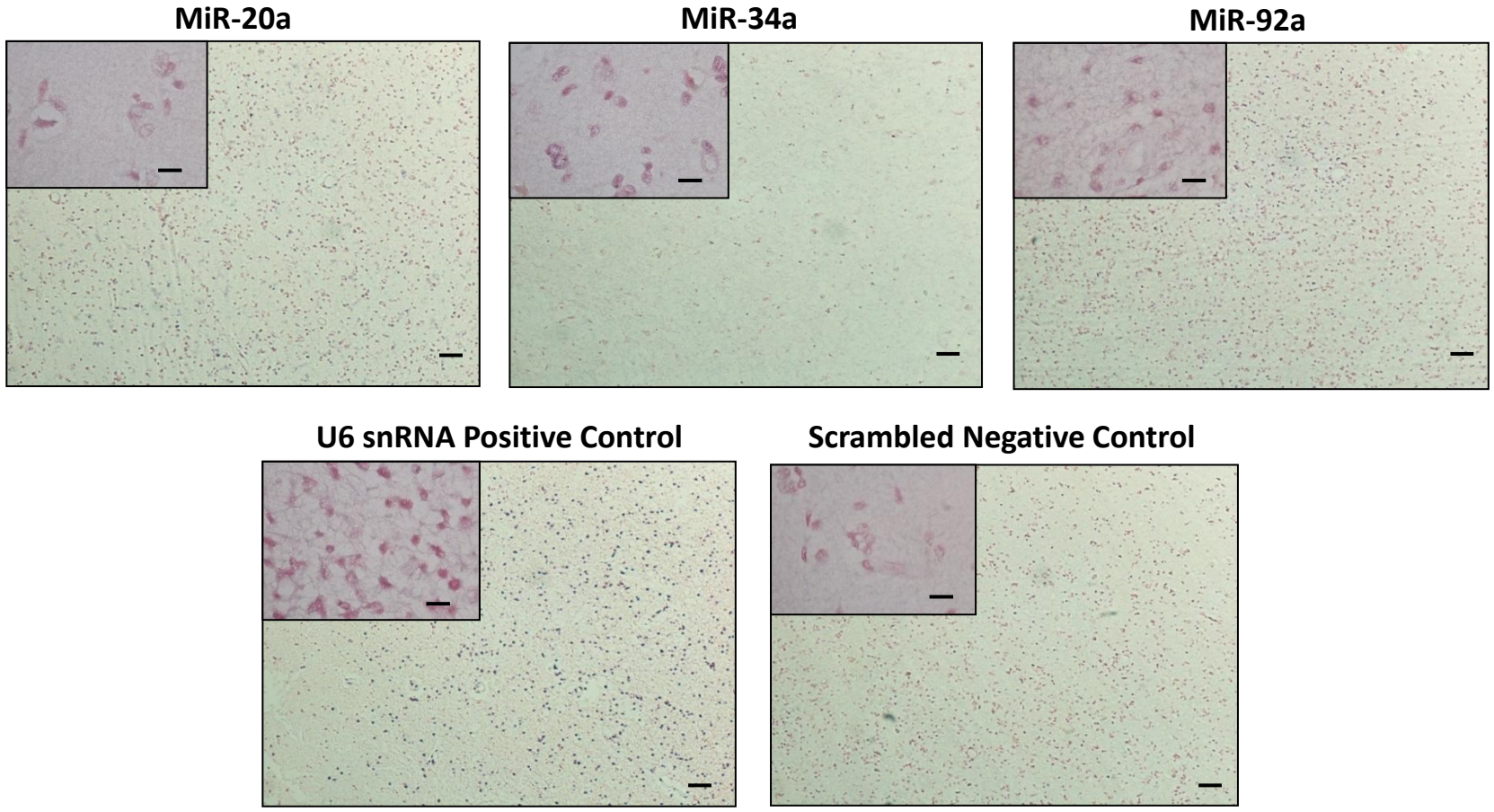


Figure 5.23 Optimisation of ISH protocol using one hour antibody incubation and two hours NBT-BCIP incubation. One hour incubation with antibody at 37 °C and two hours incubation with NBT-BCIP at 37 °C resulted in positive U6 signal and negative scrambled probe signal and positive target miRNA staining. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

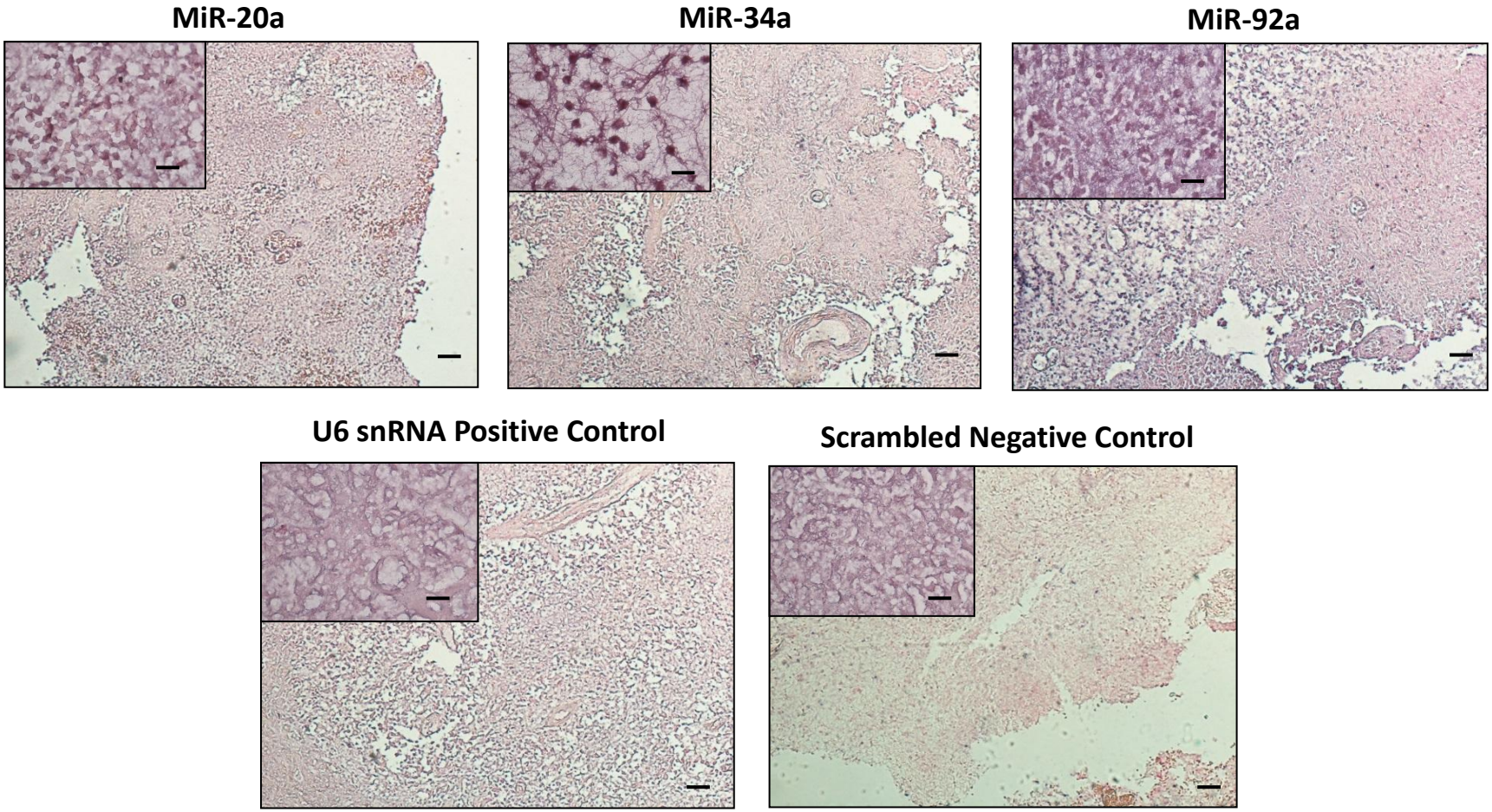


Figure 5.24 Optimisation of ISH protocol with two hours antibody and NBT-BCIP incubation. Incubation of tissue sections for two hours each at 37 °C with antibody and NBT-BCIP substrate resulted in extensive non-specific staining for all probes. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

ISH of miR-20a on female glioblastoma patients showed positive signal for three of the five viable tissue sections (Figure 5.29A). One section from each of the three female glioblastoma age groups exhibited a positive signal, BTNW 429, BTNW 871 and BTNW 1186. Expression of miR-20a in female glioblastoma patient tissue from the 20-39 and 40-59 age groups appeared to be uniform across the majority of the section, with staining mainly localised to the cytoplasm of cells (Figure 5.29A). Expression of miR-20a in the female 60+ patient tissue section also displayed signal in the cytoplasm of cells but also relatively strong signal in the nucleus also. Expression of miR-20a determined by qPCR for BTNW 429 and BTNW 1186 showed that miR-20a expression in the tissue lysate of these two patients was not significantly different ($p > 0.05$) to that of the matched serum samples and positive ISH signals were obtained for BTNW 429 and BTNW 1186 (Figure 5.29B). As matched tissue lysate was not available for any of the female tissue sections which displayed a negative ISH signal, miR-20a expression was not able to be determined using qPCR.

Four of the six tissue sections obtained from male glioblastoma patients showed positive signal for the miR-20a probe, BTNW 850, BTNW 816, BTNW 758 and BTNW 1078 (Figure 5.30A). One section from both the 20-39, BTNW 850, and 60+ age groups, BTNW 1078, showed positive signal and both sections from the 40-59 age group displayed positive signal. Three of the four positive tissue sections, BTNW 850, BTNW 758 and BTNW 1078, exhibited both nuclear and cytoplasmic signal, however BTNW 816, a male glioblastoma patient aged between 40 and 59 years, displayed cytoplasmic signal with no nuclear staining (Figure 5.30A). Expression of miR-20a determined by qPCR of tissue lysates showed a significantly higher ($p < 0.01$) expression of miR-20a in the tissue of BTNW 816 compared to serum expression and no significant difference ($p > 0.05$) in expression of miR-20a in matched tissue lysate and serum samples from BTNW 1078 and 1019 (Figure 5.30B). BTNW 816 displayed localised ISH signal rather than uniform expression across the tissue section whereas BTNW 1019 and 1078 both displayed uniform cytoplasmic and nuclear staining across the tissue section (Figure 5.30B).

ISH of the miR-34a probe in female glioblastoma patients displayed negative signal for the 20-39 age group (Figure 5.31A). The female 40-59 tissue sections displayed positive signal for both tissue sections and the viable female 60+ patient tissue section, BTNW

1186, displayed positive signal also which appeared to be stronger than that of the 40-59 age group sections (Figure 5.31A). Positive staining for miR-34a was uniform across the section with both cytoplasmic and nuclear signal of a similar strength (Figure 5.31A). Expression analysis of miR-34a by qPCR for BTNW 429 showed an absence of miR-34a expression in the tissue lysate and ISH for the BTNW 429 was also negative (Figure 5.31B). Expression of miR-34a determined by qPCR for BTNW 1186 showed a significantly lower expression ($p < 0.001$) of miR-34a in the tissue sample compared to the serum and miR-34a ISH for BTNW 1186 displayed a weak positive signal (Figure 5.31B).

Mir-34a ISH for male glioblastoma patient tissue sections showed positive signal for four of the six sections (Figure 5.32A). BTNW 850, a male glioblastoma patient aged between 20 and 39 years, displayed positive signal. Both male glioblastoma patients in the 40-59 age group and one male 60+ glioblastoma patient, BTNW 1019, exhibited positive signal with the miR-34a probe (Figure 5.32A). Two of the four positive sections, BTNW 850 and BTNW 785, showed strong nuclear signal for miR-34a compared to the cytoplasmic staining. BTNW 816 showed positive cytoplasmic staining around blood vessels with no nuclear signal. MiR-34a ISH for BTNW 816, BTNW 1078 and BTNW 1019 all displayed a weak positive signal. (Figure 5.32A). Expression of miR-34a for matched male tissue lysate samples determined by qPCR showed significantly lower ($p < 0.01$ and < 0.05) expression of miR-34a compared to serum miR-34a expression for all three patients (Figure 5.32B).

ISH of miR-92a for female glioblastoma patients showed negative signal for all but two of the tissue sections, BTNW 1186 and BTNW 743 (Figure 5.33A). BTNW 1186 displayed a positive miR-92a ISH signal however tissue lysate miR-92a expression determined by qPCR exhibited a significantly lower expression of miR-92a compared to serum expression (Figure 5.33B). MiR-92a ISH for BTNW 429 showed a negative signal for this miRNA and tissue lysate expression of miR-92a determined by qPCR exhibited a significantly lower expression of miR-92a compared to serum expression (Figure 5.33A and B).

ISH of miR-92a for male glioblastoma patients exhibited positive staining for four patients BTNW 448, BTNW 850, BTNW 758 and BTNW 1078 (Figure 5.34A). BTNW 758

displayed positive cytoplasmic miR-92a signal, BTNW 850 and BTNW 1078 displayed both positive cytoplasmic and nuclear staining (Figure 5.34A). Expression analysis of miR-92a by qPCR showed significant down-regulation of miR-92a in tissue lysate samples compared to serum samples for BTNW 816, BTNW 1078 and BTNW 1019 (Figure 5.34B).

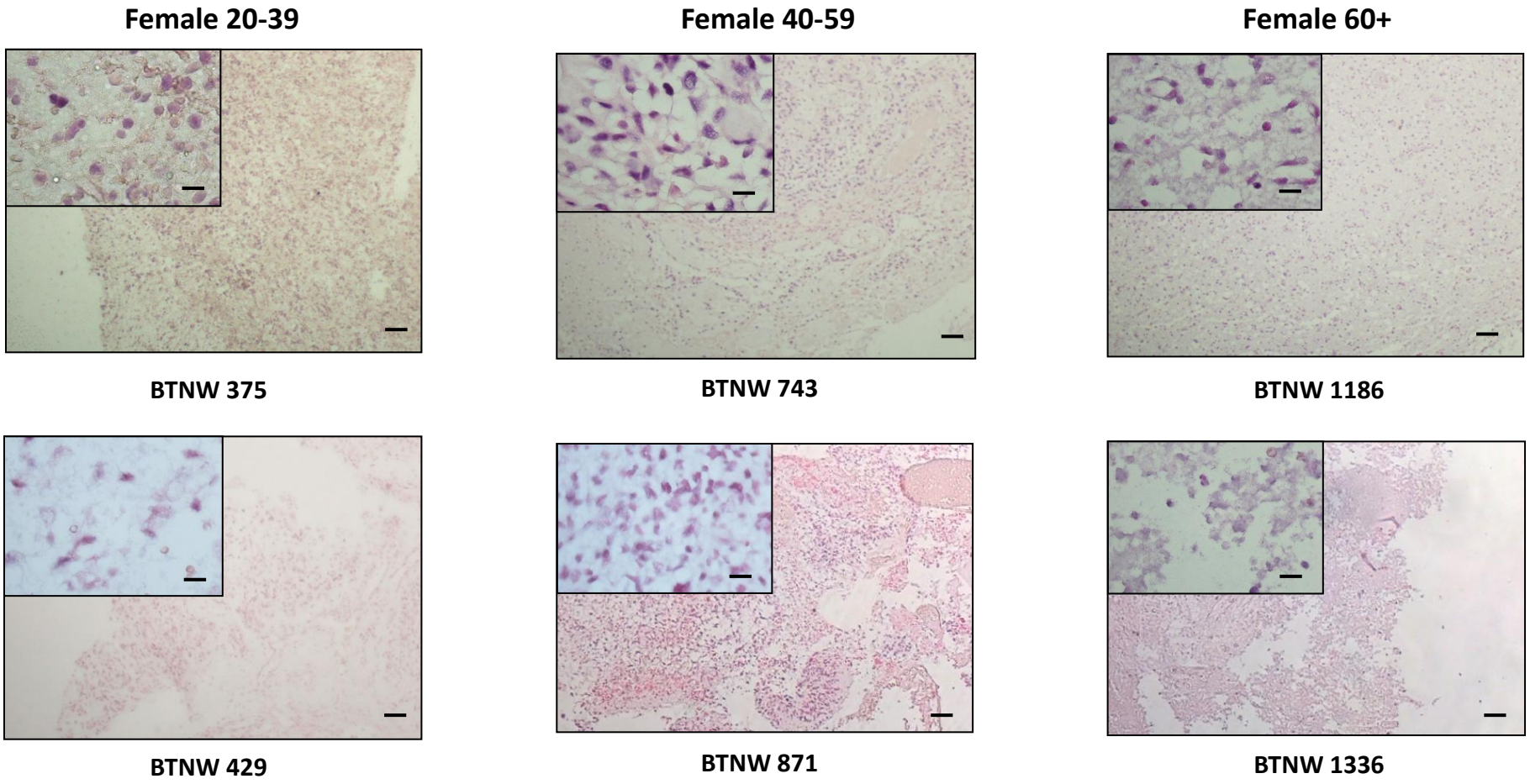


Figure 5.25 ISH of U6 snRNA positive control in female glioblastoma patient tissue sections. All six female patient tissue sections displayed positive signal for the U6 snRNA positive control probe. BTNW 1336 showed extensive necrosis within the tissue section resulting in poor signal. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

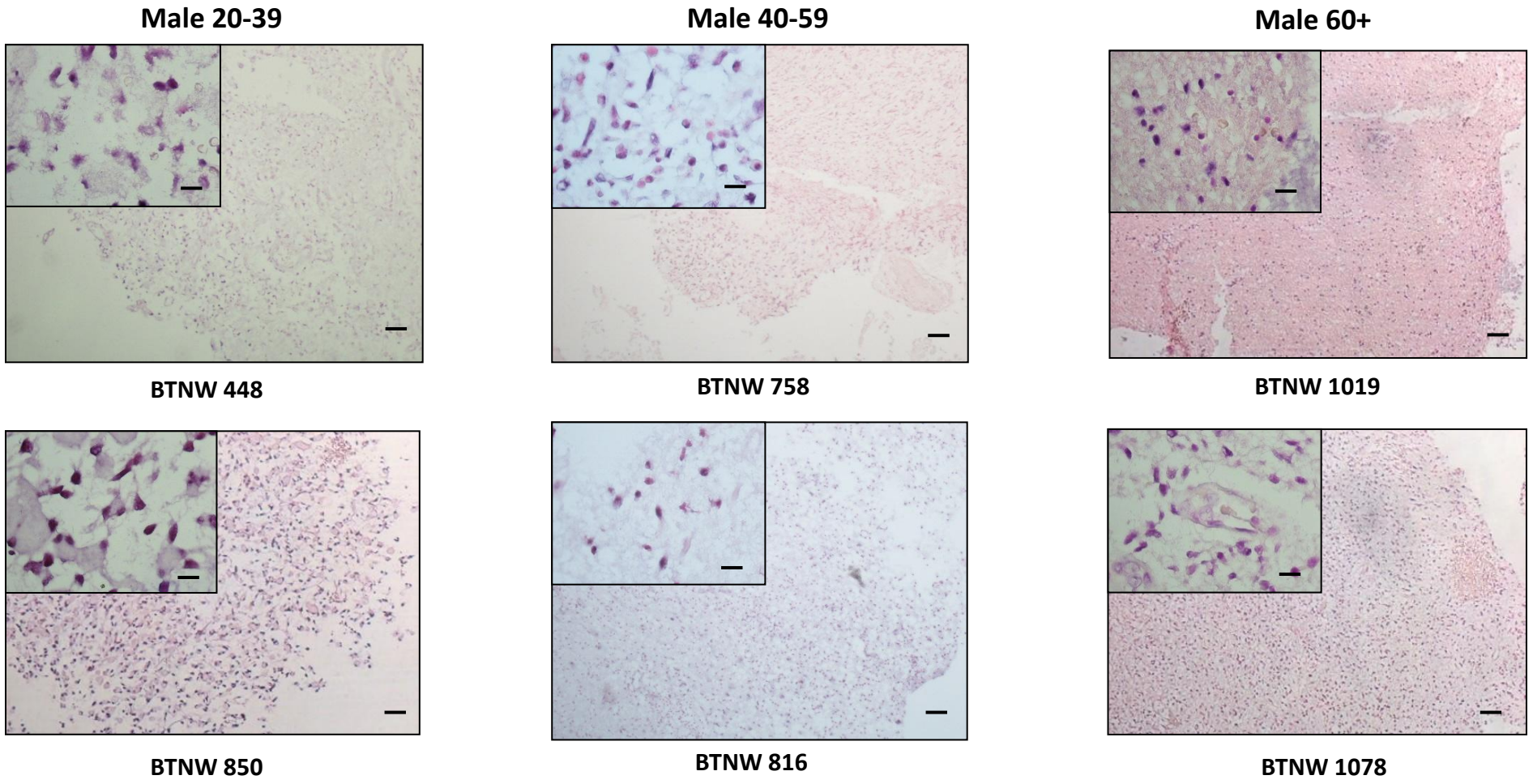


Figure 5.26 ISH of U6 snRNA positive control in male glioblastoma patient tissue sections. All six male patient tissue sections displayed positive signal for the U6 snRNA positive control probe. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm, inset 1µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

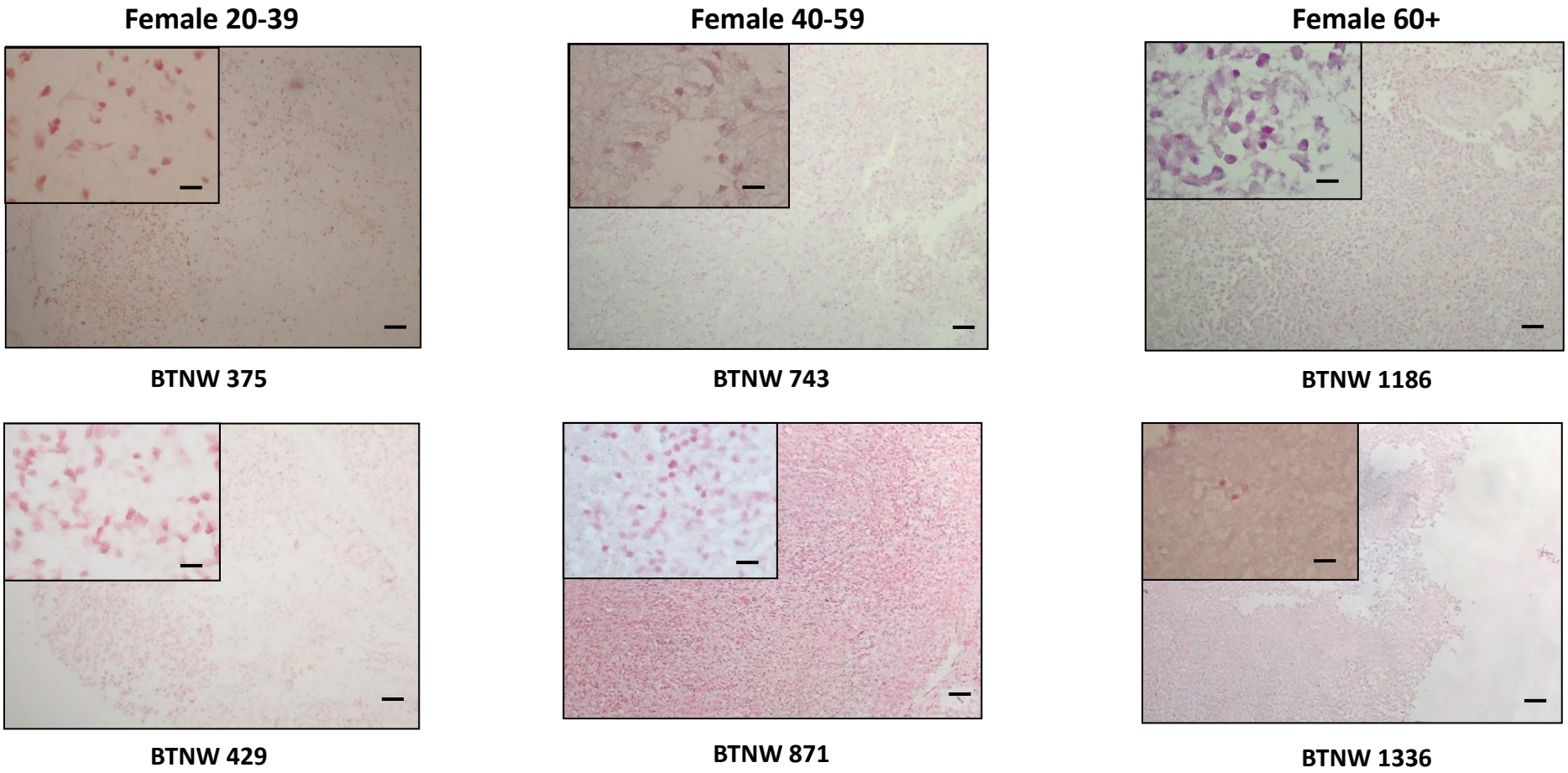


Figure 5.27 ISH of scrambled negative control probe on female glioblastoma patients. All female glioblastoma patient tissue sections showed no probe signal for the negative control scrambled probe. BTNW 1336 showed extensive necrosis within the tissue section resulting in poor signal. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

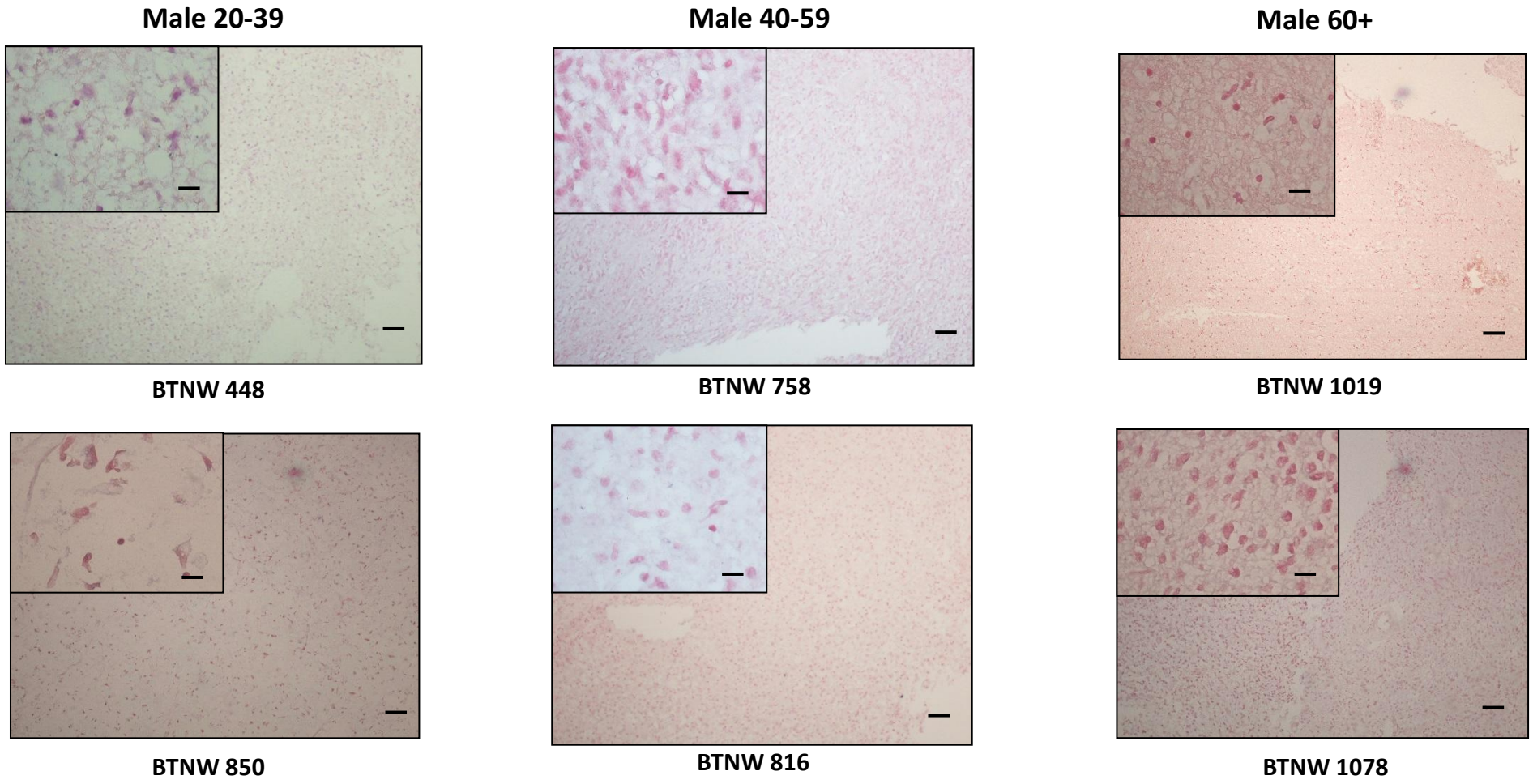


Figure 5.28 ISH of scrambled negative control probe on male glioblastoma patients. All male glioblastoma patient tissue sections showed no probe signal for the negative control scrambled probe. Main images x 10 magnification, inset x 100 magnification. Scale bars – 10 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

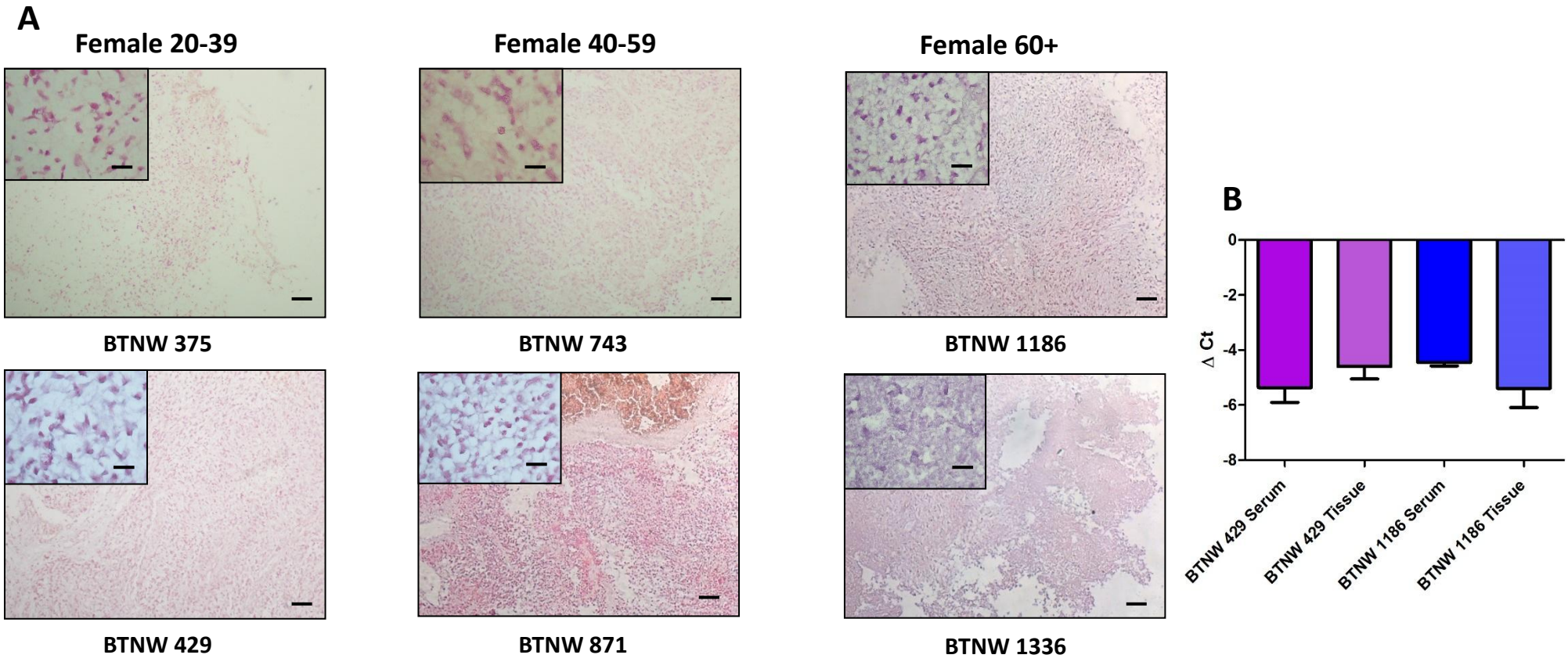


Figure 5.29 MiR-20a ISH of female glioblastoma patient tissue sections and qPCR of matched frozen tissue samples. A) Positive signal for miR-20a was obtained for three of the five viable female tissue sections in each of the three age groups. MiR-20a signal was observed to be distributed both in the nucleus and cytoplasm of cells within the sections. B) Comparison of tissue and serum miR-20a expression determined by qPCR demonstrated no signficant difference in miR-20a expression between matched tissue lysate and serum samples for BTNW 429 and 1186 who also demonstrated positive miR-20a ISH signals. Data shown as mean of replicates (n = 3) plus SD, analysed by Mann-Whitney U test, $p > 0.05$. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

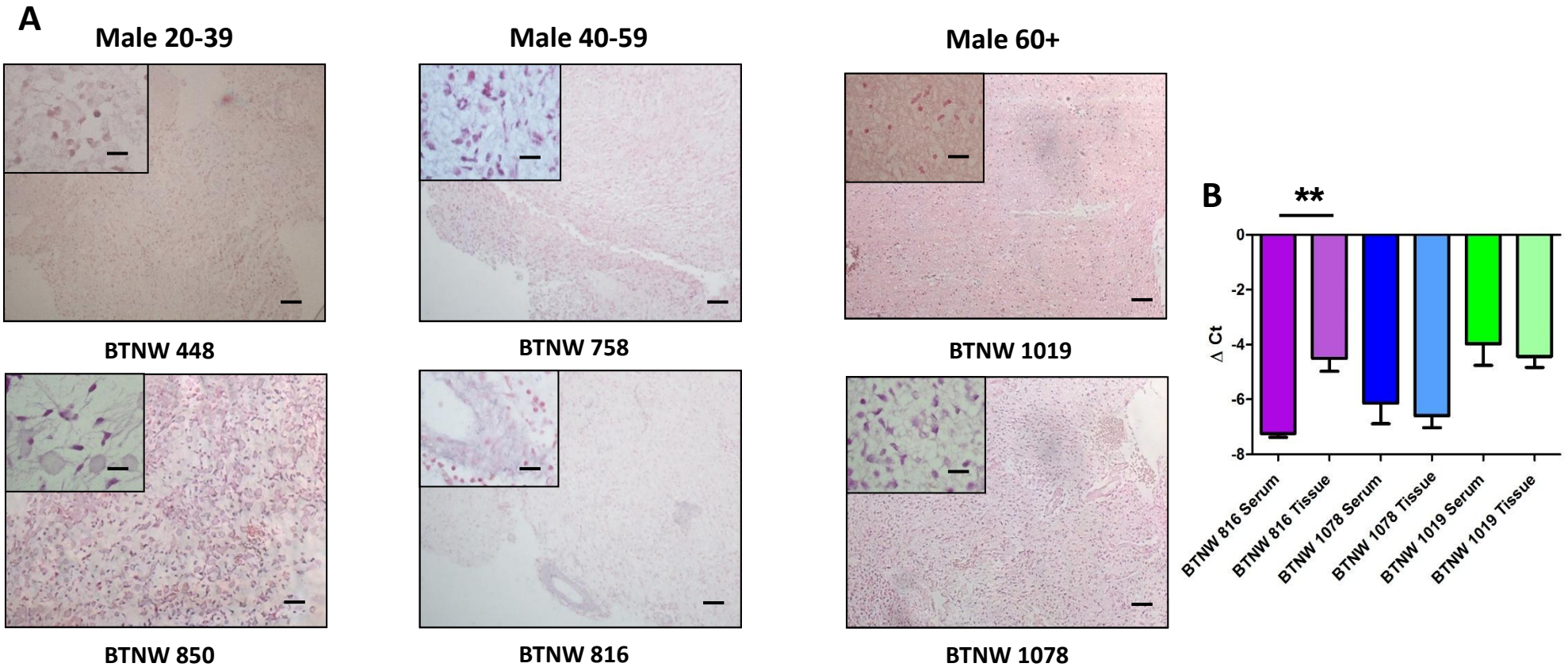


Figure 5.30 MiR-20a ISH of male glioblastoma patient tissue sections and qPCR data of matched frozen tissue samples. A) Positive signal of miR-20a was observed for four of the six tissue sections, miR-20a signal was observed to be strongest in the nucleus of cells in three of the sections however cytoplasmic staining was also observed, BTNW 816 displayed cytoplasmic staining of miR-20a only. B) Comparison of ISH and qPCR miR-20a expression data showed no significant difference in expression of miR-20a for two of the three matched tissue lysate samples compared to matched serum samples. BTNW 816 showed a significantly higher expression of miR-20a in the tissue lysate compared to matched serum. Data shown as mean of replicates (n = 3) plus SD, analysed by Mann-Whitney U test. Significance between groups indicated by a solid black line, $^{**}p < 0.01$. Scale bars – 10 µm, 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

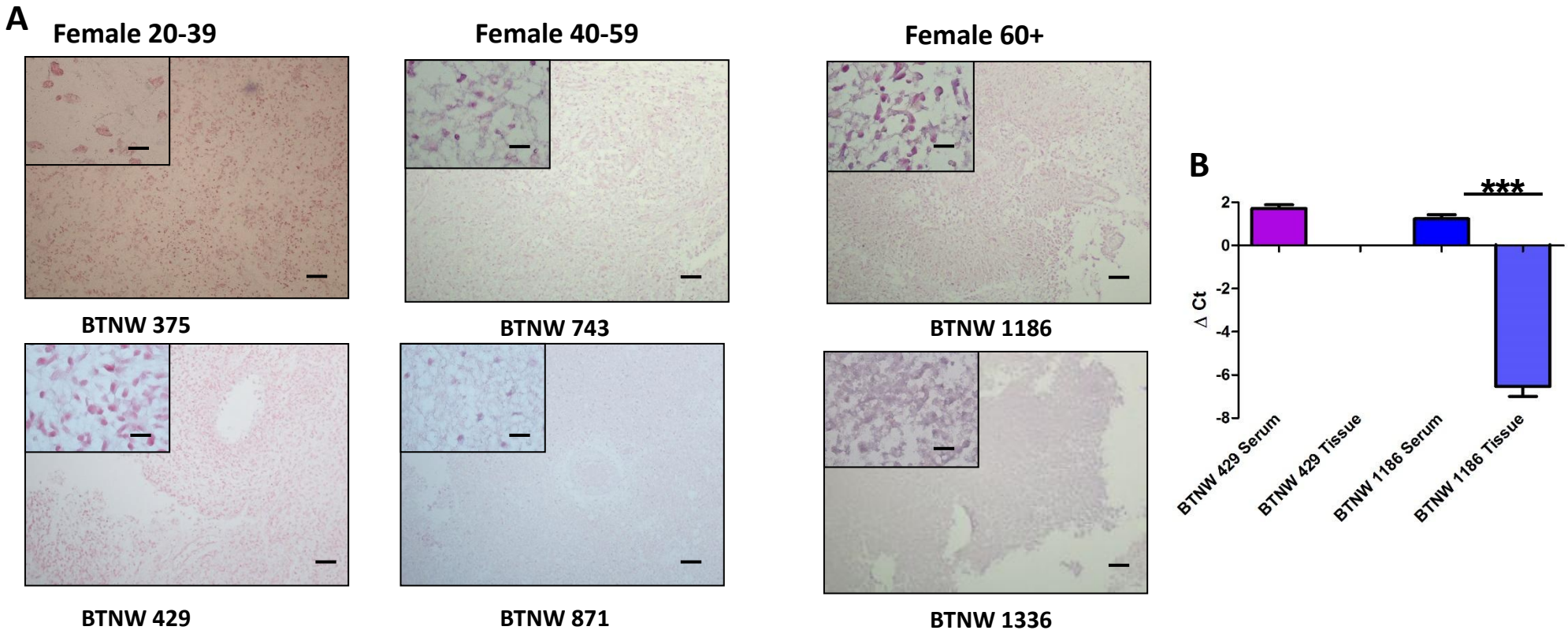


Figure 5.31 MiR-34a ISH of female glioblastoma patient tissue sections and qPCR data from matched frozen tissue samples. A) Positive signals for miR-34a were obtained for three of the viable female tissue sections, BTNW 743, BTNW 871 and BTNW 1186. MiR-34a ISH signal showed strong nuclear staining and weak cytoplasmic staining. B) Comparison of ISH and qPCR data showed that BTNW 1186 tissue lysate miR-34a expression was significantly lower compared to matched serum expression, BTNW 1186 showed a weak miR-34a ISH signal. MiR-34a was not detected in the matched tissue lysate sample for BTNW 429 and ISH miR-34a signal for this patient was negative. Data shown as mean of replicates (n = 3) plus SD, analysed by Mann-Whitney U test. Significance between groups indicated by a solid black line, ***$p < 0.001$. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

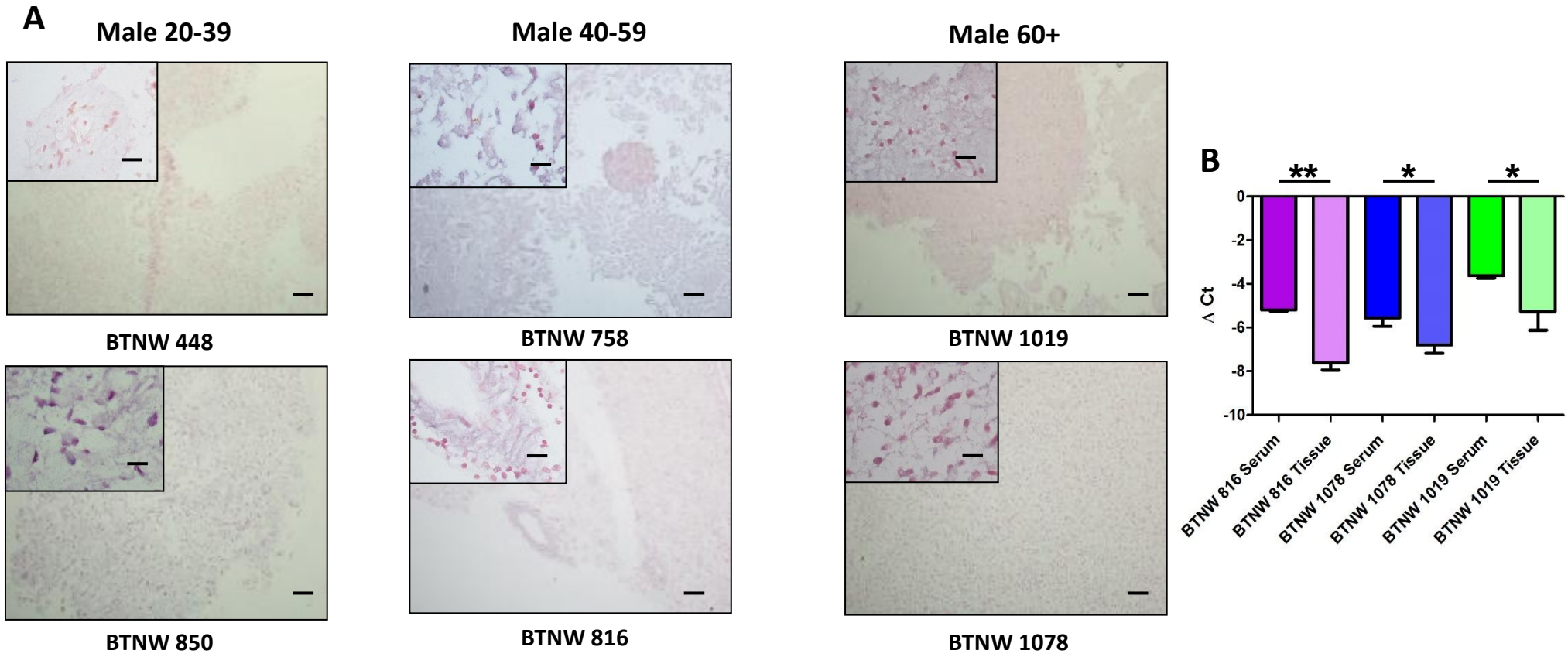


Figure 5.32 MiR-34a ISH of male glioblastoma patient tissue sections and qPCR data for matched frozen tissue samples. A) Positive signals were obtained for five of the six male tissue sections. MiR-34a was observed to be enriched in the nucleus of cells demonstrated by a strong signal and a weaker cytoplasmic signal. B) Comparison of miR-34a ISH and qPCR expression showed a down-regulation of miR-34a expression in tissue lysate samples for all three patients compared to matched serum samples. Data shown as mean of replicates (n = 3) plus SD, analysed by Mann-Whitney U test. Significance indicated between groups by solid black line, *$p < 0.05$, **$p < 0.01$. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

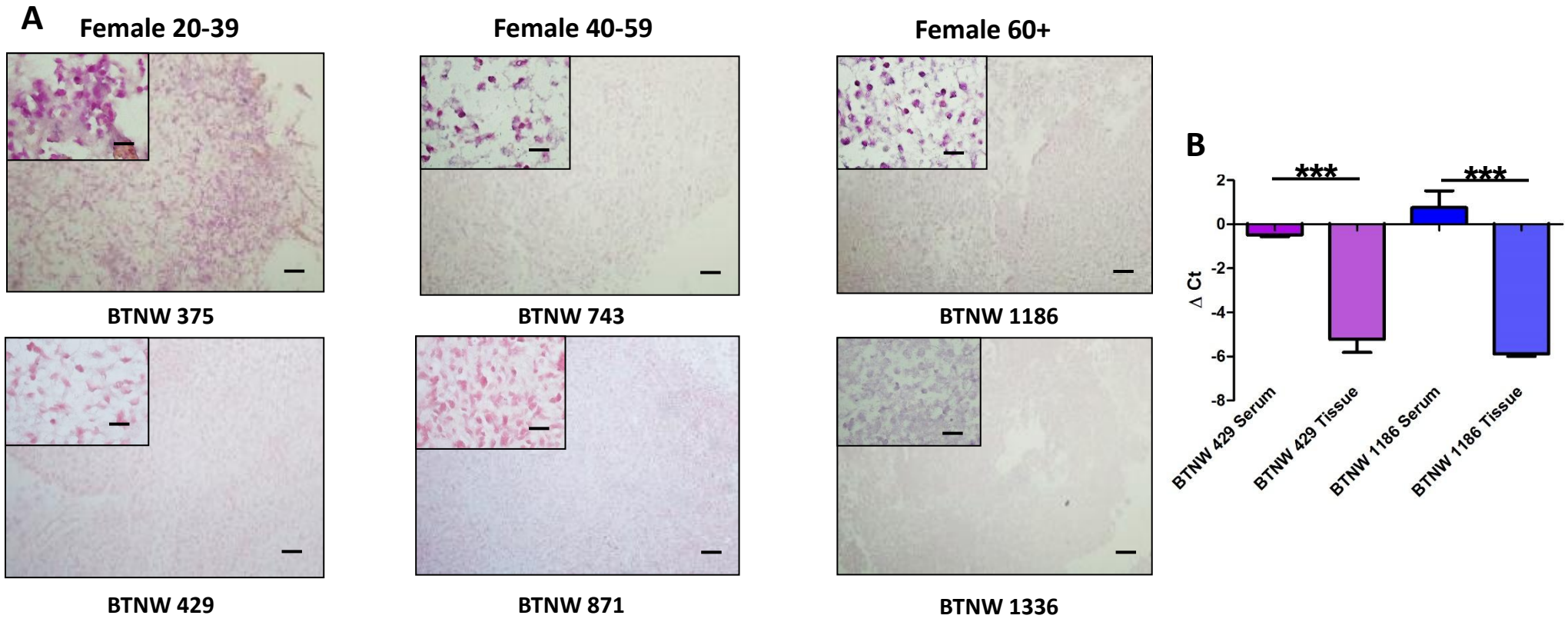


Figure 5.33 MiR-92a ISH of female glioblastoma patient tissue sections and qPCR data from matched frozen tissue samples. A) A positive miR-92a signal was observed for two of the five viable female tissue sections, BTNW 1186 and BTNW 743 with the remaining four displaying negative signals. Positive staining for BTNW 1186 was enriched in the nucleus of cells within the section. B) Comparison of ISH and qPCR data for miR-92a expression showed a down-regulation of miR-92a in the tissue lysate sample compared to matched serum and signal for BTNW 429 ISH was negative. Tissue lysate miR-92a expression was down-regulated compared to the serum of BTNW 1186 which displayed a positive ISH signal. Data shown as mean of replicates (n = 3) plus SD, analysed by Mann-Whitney U test. Significance indicated between groups by a solid black line, ***$p < 0.001$. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

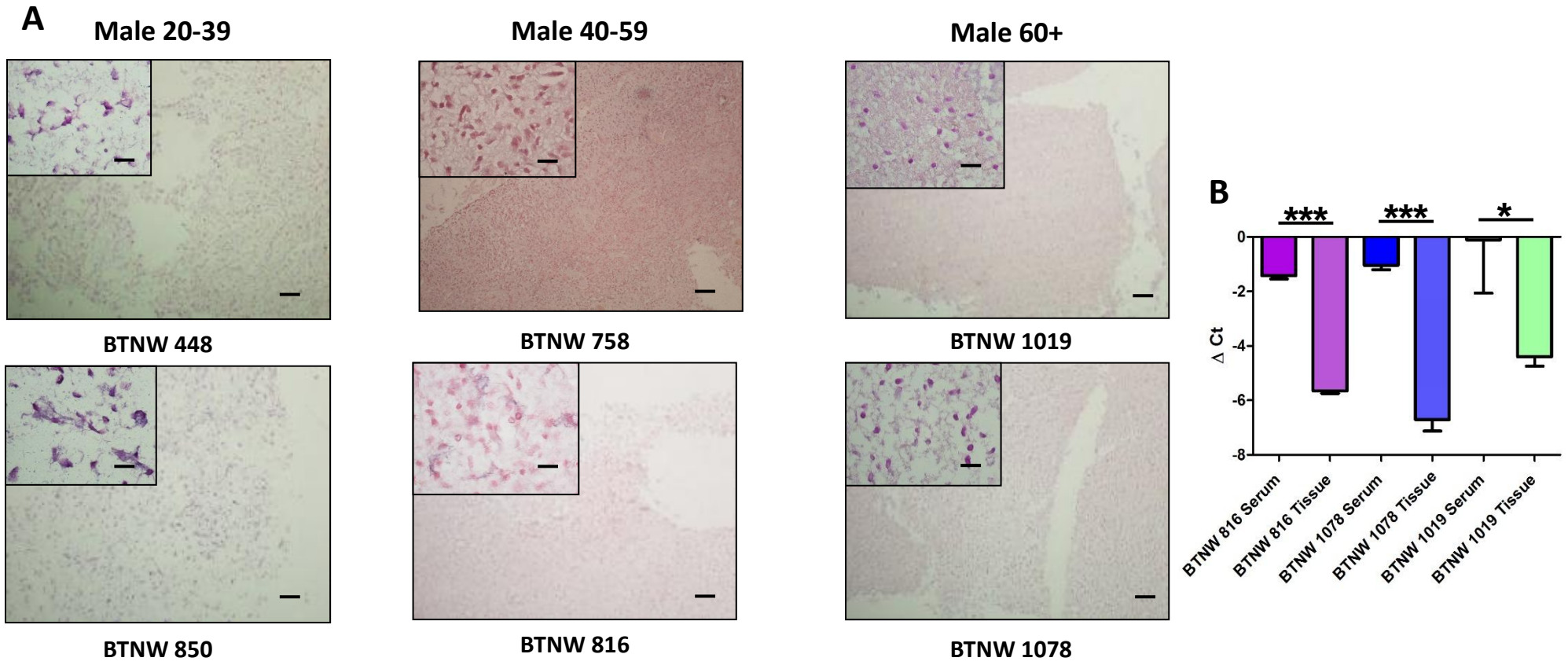


Figure 5.34 MiR-92a ISH of male glioblastoma patient tissue sections and qPCR data from matched frozen tissue samples. A) Positive miR-92a ISH signals were observed for four of the six male tissue sections BTNW 448, BTNW 850, BTNW 758 and BTNW 1078, with nuclear enrichment in three of the four positive sections except BTNW 758. B) Comparison of ISH and qPCR miR-92a expression showed signficantly lower expression in tissue lysate miR-92a expression compared to matched serum samples in all three patients. Data shown as mean of replicates (n = 3), plus SD, analysed by Mann-Whitney U test. Significance between groups indicated by a solid black line, *$p < 0.05$, ***$p < 0.001$. Scale bars – 10 µm, inset 1 µm. Blue signal – miRNA target, red signal – nuclear fast red counterstain.

5.2.6 Expression of Serum Biomarkers in Cerebrospinal Fluid

The use of serum as a relatively non-invasive test for glioblastoma biomarkers is attractive however the proximity of cerebrospinal fluid to the location of glial tumours may provide a more accurate representation of glioblastoma miRNA expression. Expression of the serum miRNA biomarkers identified in Section 4.2.4, in cerebrospinal fluid (CSF), from four glioblastoma patients and six patients with other neurological disorders (Table 5.2), was measured to determine whether there were similarities with serum miRNA expression. If expression of the serum miRNAs was similar it may suggest that CSF could be a more useful and accurate means of diagnosing and predicting prognosis for glioblastoma patients. Although found to be not significantly altered in expression in the validation study, miR-150 was included in the CSF study to determine whether expression may be significantly altered in an alternative sample type.

Table 5.2 Neurological disorders of patient CSF samples utilised as controls.

Patient Number	Neurological Disorder
1208	Dermoid Cyst
1289	Normal Pressure Hydrocephalus
1293	Dermoid Cyst
1210	Hydrocephalus
1234	Normal Pressure Hydrocephalus
1238	Normal Pressure Hydrocephalus

MiR-20a and miR-92a were detected in the CSF of glioblastoma patients however, miR-30c, miR-34a and miR-150 were undetected. MiR-20a showed higher expression in the CSF of glioblastoma patients aged between 40 and 39 years and reduced expression in glioblastoma patients aged over 60 years (Figure 5.32). CSF samples from glioblastoma patients aged between 20 and 39 years were not available for this study. MiR-92a expression was significantly higher ($p < 0.001$) in the CSF of male glioblastoma patients compared to sex matched controls (Figure 5.33).

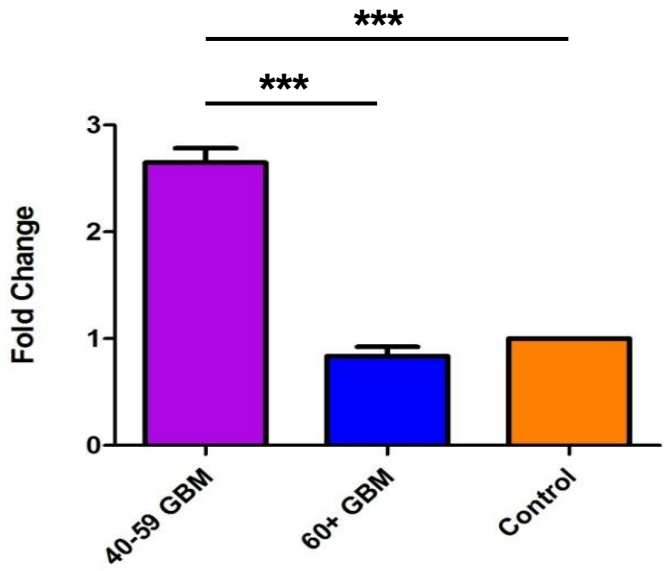


Figure 5.35 Expression of miR-20a in the cerebrospinal fluid of glioblastoma patients grouped by age. CSF from glioblastoma patients aged between 40 and 59 years exhibited a higher expression of miR-20a compared to CSF obtained from glioblastoma patients over the age of 60 years and age matched controls. There was no significant difference in miR-20a expression between glioblastoma patients over the age of 60 years and age matched controls. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by one way ANOVA with Tukey *post-hoc* test. Significance between groups indicated by a solid black line, ***$p < 0.001$.

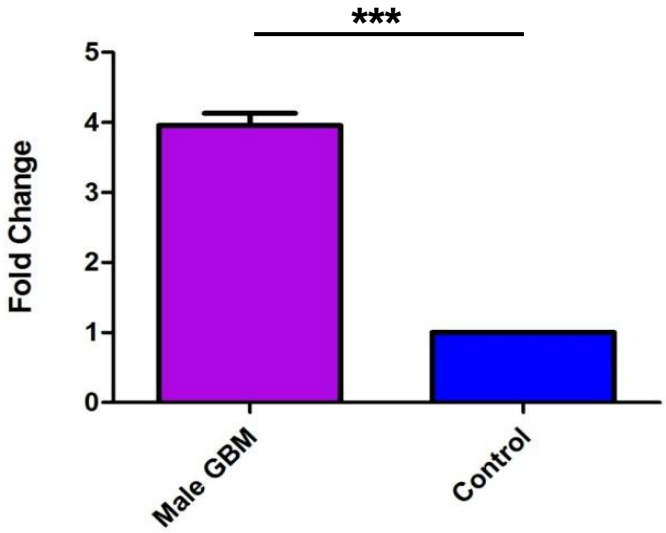


Figure 5.36 Expression of miR-92a in the cerebrospinal fluid of glioblastoma patients grouped by gender. MiR-92a expression was significantly higher in the CSF of male glioblastoma patients compared to gender matched controls. Data shown as mean of replicates (n = 3 per group) plus SD, analysed by unpaired student's t-test, ***$p < 0.001$.

5.2.7 MicroRNA Expression of Lymphocytes

To further determine whether the miRNA biomarkers detected within the serum of glioblastoma patients originated from a source other than the glioblastoma microenvironment, the expression of miR-20a, 30c, 34a and 92a was measured in lymphocytes obtained from glioblastoma patients. The buffy coat containing lymphocytes was collected from fresh blood samples from four glioblastoma patients from the Brain Tumour North West tissue bank upon diagnosis and analysed using qPCR. Due to the unavailability of non-cancerous control blood samples three control patients, two low-grade glioma and one gliosarcoma were used for this study. As a result of the small sample size, patients were not grouped by age or gender for analysis. Analysis of the four serum miRNA biomarkers in lymphocytes showed no significant difference (p > 0.05) in expression between glioblastoma patients and control patients (Figure 5.34).

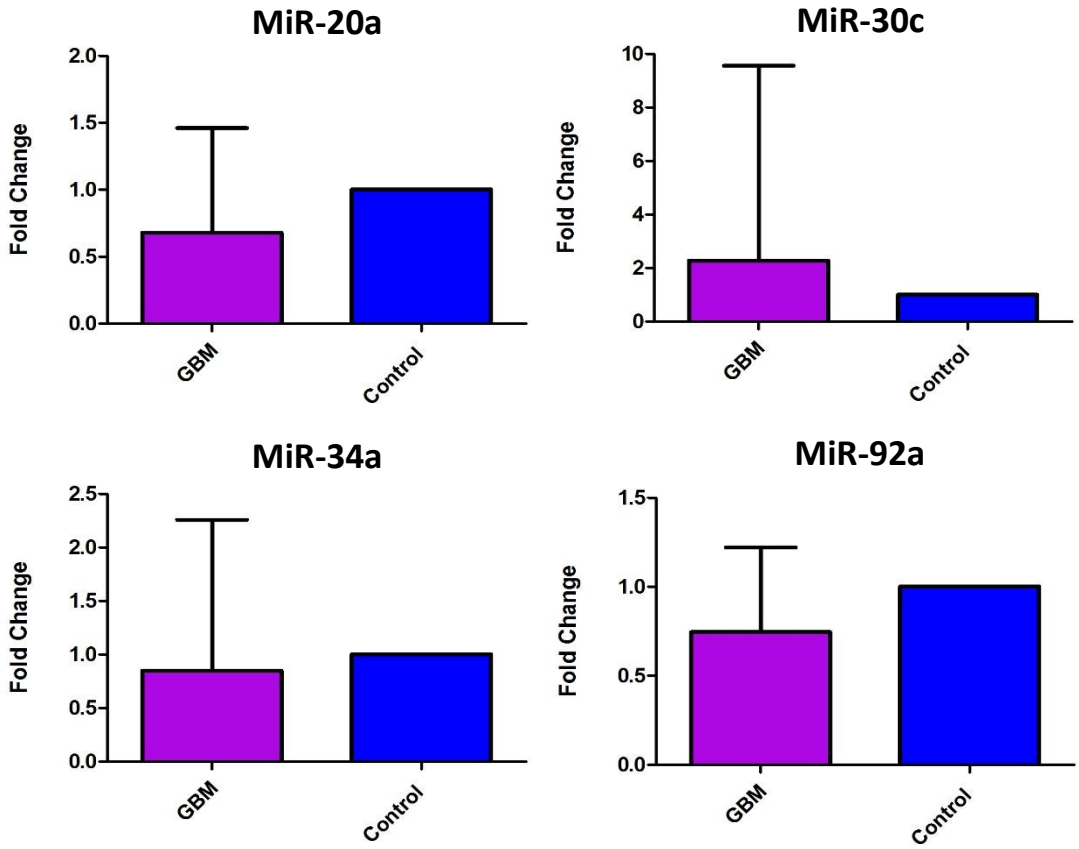


Figure 5.37 Expression of serum miRNA biomarkers in lymphocytes. Expression of the four serum miRNA biomarkers was not significantly different in lymphocytes obtained from glioblastoma patients compared to those obtained from control patients. Data shown as mean of replicates (n = 4 glioblastoma patients, n = 3 control patients) analysed by Mann-Whitney U test, p > 0.05.

5.3 Discussion

Following the identification and validation of four miRNA biomarkers in the serum of glioblastoma patients, the human serum cell culture model developed in Chapter 3 was used to investigate the origin of the serum miRNA biomarkers and investigate the effect of patient serum on the expression of miRNA biomarkers in U87MG and SVGp12 cells. Initial studies were performed to compare the expression of the miRNA biomarkers in pools of serum samples from glioblastoma and control patients to the expression of the miRNAs in cell lines cultured in 10 % FBS. Expression of the miRNA biomarkers in the glioblastoma and control serum pools were compared to ensure that the initial expression of the miRNAs in the serum reflected the expression determined in Section 4.2.4 and 4.2.5. Subsequent studies were performed to determine the effect of replacing FBS with patient serum on the biomarker expression in the cell lines.

MiR-34a expression was not significantly different in the 60+ glioblastoma patient serum pool compared to the 60+ control patient serum pool. MiR-34a expression in 60+ glioblastoma patient and 60+ control patient serum pools was significantly lower compared to miR-34a expression in both U87MG and SVGp12 cell lines cultured in 10 % FBS. This could be as a result of the lower expression of miRNA observed in the serum of patients compared to tissue expression (Skog et al., 2008). Comparison of the 60+ glioblastoma serum pool to U87MG cells cultured in FBS showed no significant difference between intracellular and extracellular expression. MiR-34a expression in the 60+ glioblastoma patient pool showed the greatest significant decrease compared to the spent media of U87MG cells. This significant decrease was also observed between the 60+ glioblastoma pool and intracellular U87MG expression but was not as large. This suggests that miR-34a expression in U87MG cells cultured in 10 % FBS is significantly higher in exosomes within the media compared to miR-34a expression within the cells. This is similar to the high expression of miR-34a observed in the serum in Section 4.2.4 and 4.2.5 in this thesis and the down-regulation of miR-34a in glioblastoma tissue reported by other studies (Gao et al., 2013, Genovese et al., 2012, Li et al., 2011a). MiR-34a expression in the 60+ glioblastoma patient serum pool was significantly lower than SVGp12 expression extracellularly. No significant difference was observed between intracellular and extracellular SVGp12 expression cultured in 10 % FBS, suggesting that miR-34a expression is not significantly different between

exosomes in the media and expression within SVGp12 cells. MiR-34a expression in the 60+ glioblastoma patient serum pool was significantly lower compared to intracellular SVGp12 expression than intracellular U87MG expression. This suggests that intracellular miR-34a expression in U87MG cells is lower than miR-34a expression in SVGp12 cells. This is again in accordance with previous reports of miR-34a down-regulation in the tissue of glioblastoma patients (Li et al., 2011a, Li et al., 2009).

When cultured in 60+ glioblastoma patient serum, intracellular miR-34a expression in U87MG cells was significantly higher compared to U87MG cells cultured in 10 % FBS. MiR-34a expression in U87MG cells intracellularly was significantly higher compared to extracellular U87MG expression and SVGp12 expression. Extracellular U87MG miR-34a expression and SVGp12 miR-34a expression, intracellular and extracellular was not significantly different when cultured in 10 % 60+ glioblastoma serum compared to culture in 10 % FBS. This trend was also observed in cells cultured in 10 % 60+ control patient serum and comparison of intracellular U87MG miR-34a expression showed no significant difference in expression between cells cultured in 60+ glioblastoma patient serum and 60+ control patient serum.

U87MG expresses wild-type p53 and miR-34a is a transcriptional target of p53 and is part of a positive feedback loop which results in up-regulation of miR-34a (Okada et al., 2014). The increase in miR-34a expression in U87MG cells cultured in patient serum may be due to an increase in miR-34a taken up by U87MG cells from the patient serum resulting in a cumulative increase in expression of this miRNA compared to U87MG cells cultured in FBS (Okada et al., 2014). Expression of miR-34a in SVGp12 cells however, was not significantly different between those cultured in the 60+ patient serum pools and those cultured in FBS. SVGp12 cells also express wild-type p53 (Henriksen et al., 2014b), therefore if the up-regulation of miR-34a in U87MG cells was a result of the positive feedback loop between miR-34a and p53, a similar increase would be expected in SVGp12 cells. Furthermore the intracellular increase in miR-34a expression in U87MG cells was observed in cells cultured in both 60+ glioblastoma patient serum and 60+ control patient serum, and the increase was not significantly different between the serum pools. As miR-34a expression was determined to be higher in the 60+ glioblastoma patient serum pool than the 60+ control patient serum pool, a greater increase in U87MG cells culture in the glioblastoma pool would be

expected if the increase was related to the positive feedback loop, therefore alternative factors are likely to be the cause of the increase in expression that was observed.

Comparison of miR-92a expression in pooled male glioblastoma serum and pooled male control serum showed that miR-92a expression was significantly higher in male glioblastoma serum compared to male control patient serum as observed in Sections 4.2.4 and 4.2.5. MiR-92a expression in both male patient serum pools was significantly lower compared to miR-92a expression in U87MG and SVGp12 cells cultured in 10 % FBS. Again, this is likely to be due to the lower expression of miRNA in exosomes within the circulation compared to tissue (Skog et al., 2008). MiR-92a expression in the male control serum pool compared to intracellular SVGp12 expression showed the most significant difference in expression than extracellular SVGp12 expression and intracellular U87MG expression. This suggests that intracellular SVGp12 miR-92a expression is higher than that of exosomes in the media of SVGp12 cells as well as U87MG cells.

MiR-92a expression of U87MG cells cultured in 10 % male glioblastoma serum and 10 % male control serum showed no significant difference compared to those cultured in 10 % FBS. Intracellular miR-92a expression of SVGp12 cells cultured in 10 % male glioblastoma serum was significantly higher compared to those cultured in 10 % FBS. Intracellular miR-92a expression of SVGp12 cells cultured in 10 % male control patient serum showed no significant difference in expression compared to FBS. This suggests that exosomes containing a higher expression of miR-92a in male glioblastoma patient serum could be taken up by non-cancerous cells causing an increase in intracellular miR-92a. MiR-92a has been shown to target CDH1, a transmembrane glycoprotein which promotes cell-cell adhesion of epithelial cells the loss of which is a key hallmark of the epithelial-mesenchymal transition (Ding, 2014). Increased miR-92a expression in recipient non-cancerous cells could down-regulate expression of CDH1 reducing cell-cell adhesion and promoting cell motility and invasiveness (Ding, 2014), thereby promoting glioblastoma growth and invasion.

MiR-20a and 30c were detected intracellularly alone in U87MG cells cultured in 10 % FBS only. The absence of expression of these two miRNAs in SVGp12 cells both

intracellularly and extracellularly may be due to these miRNAs not being expressed by this particular cell line or being expressed below the limit of detection by qPCR. The lack of U87MG extracellular expression of these two miRNAs may suggest that these particular miRNAs are not exported from U87MG cells cultured in standard FBS conditions which may be due to the selectivity of exosomal packaging (Chen et al., 2012).

Comparison of miR-20a expression in glioblastoma patient and control patient serum pools showed expression was significantly higher in the glioblastoma serum pool. No significant difference in expression between miR-20a expression in glioblastoma patient serum compared to U87MG cells cultured in 10 % FBS. MiR-20a expression in control patient serum was significantly lower compared to U87MG cells cultured in 10 % FBS. As miR-20a was highly expressed in the serum of glioblastoma patients and is up-regulated in glioblastoma patient tissue (Srinivasan et al., 2011), this suggests miR-20a expression in U87MG cells is similarly up-regulated to miR-20a expression in the serum of glioblastoma patients and therefore expresses miR-20a at a higher level than control patient serum.

U87MG cells cultured in 10 % glioblastoma patient serum and 10 % control patient serum showed no significant difference in miR-20a expression compared to those cultured in 10 % FBS. This suggests that human serum does not affect miR-20a expression in U87MG cells.

Comparison of miR-30c expression in 20-39 glioblastoma patients and 20-39 control patients showed miR-30c was significantly lower in the glioblastoma patient serum pool compared to the control patient serum pool as observed in Sections 4.2.4 and 4.2.5. MiR-30c expression in the 20-39 glioblastoma patient serum pool was significantly lower compared to U87MG cells cultured in 10 % FBS. MiR-30c has been previously found to be up-regulated in TRAIL-resistant gliomas and down-regulated in TRAIL-sensitive gliomas (Quintavalle et al., 2012). The expression of miR-30c in the U87MG cell line has not previously been reported however a previous study investigating the effect of TRAIL on glioma cell lines demonstrated U87MG to be resistant to TRAIL treatment, suggesting that U87MG is a TRAIL-resistant cell line (Kim et al., 2004) which as a result, may express miR-30c at a higher level.

The culture of U87MG cells in 10 % 20-39 glioblastoma patient serum resulted in an up-regulation in expression of miR-30c compared to those cultured in 10 % FBS. No significant difference in miR-30c expression was observed between U87MG cells cultured in 10 % 20-39 control patient serum and 10 % FBS. As miR-30c was reduced in 20-39 glioblastoma patient serum, a high level of expression in U87MG cells cultured in glioblastoma serum is unlikely to be due to a positive feedback mechanism and may be due to the presence of alternative factors within the serum which may promote the expression of miR-30c.

Initial identification of miRNA expression in tissue samples obtained from glioblastoma patients over the age of 60 years showed that of the five serum biomarkers identified in Section 4.2.4, three were detected. In male 60+ glioblastoma tissue miR-92a and miR-30c showed relatively high expression whereas in female 60+ glioblastoma tissue miR-92a showed lower levels of expression. MiR-30c has previously been observed to be up-regulated in TRAIL-resistant glioblastomas (Quintavalle et al., 2012). The gender difference in male and female tissue miR-92a expression in Section 5.2.1 is similar to that observed in Section 4.2.4 in the serum data. MiR-150 was expressed in male 60+ glioblastoma tissue alone, exhibiting a higher level of expression compared to age and sex matched control tissue. MiR-150 was not detected in the serum of glioblastoma patients over the age of 60 years (Section 4.2.4), further investigation of tissue profiles in the additional age groups would allow a comparison of miR-150 expression by age in glioblastoma tissue, which could further permit elucidation of tissue expression of this miRNA compared to serum expression. The role of miR-150-5p in glioblastoma has not been investigated in previous studies, however miR-150* is reported to be down-regulated in grade II-IV glioma serum samples (Yang et al., 2013a). MiR-20a was not detected in patient tissue, which may be due to the inverse correlation of miR-20a expression and age, identified in Section 4.2.4 and also observed in the TCGA dataset in Section 5.2.2. Mir-34a was not detected in the tissue of glioblastoma patients over the age of 60 years, and yet miR-34a was detected in the serum of glioblastoma patients in this age group in Section 4.2.4 however miR-34a has previously been shown to be down-regulated in glioblastoma tissue (Yin et al., 2013) which may attribute to the absence of miR-34a in the tissue samples. Further studies investigating the expression of the three biomarkers in glioblastoma tissue from patients aged between

20 and 39 years and 40-59 years would provide a more complete analysis of serum miRNA biomarker expression in glioblastoma tissue.

Further analysis of tissue miRNA expression using microarray data from the TCGA identified all four validated serum miRNA biomarkers to be expressed in glioblastoma tissue. Contrary to published data, miR-34a was detected in the tissue of glioblastoma patients and the level of expression was correlated with age. The high expression of miR-34a in tissue analysed in the TCGA is in contrast to other studies which have identified miR-34a as being down-regulated in glioblastoma tissue thus hindering its tumour suppressive functions (Gao et al., 2013). The correlation of miR-34a expression and age in the tissue data is similar to the observed correlation of serum miR-34a expression and age in this study (Section 4.2.4) as well as in previous studies (Li et al., 2011b, Sawada et al., 2014). Patients with a higher expression of miR-34a were found to have a worse prognosis than those with a low expression of miR-34a a previous study investigating miR-34a expression and prognosis in grade III and grade IV gliomas demonstrated that patients with a high expression had a better prognosis than those with a low expression (Gao et al., 2013), this is in contrast to the TCGA data analysed in this thesis. Differences in prognosis based on miR-34a between these two studies may be due to the size of the patient groups analysed, 558 from the TCGA dataset in contrast to 79 patients analysed by Gao *et al.* (2013) however the improved survival of patients observed in the TCGA dataset in Section 5.2.2 with a reduced expression of miR-34a does not correlate with the role of this miRNA as a tumour suppressor, as the up-regulation of a tumour suppressor would suggest an improved prognosis. A previous study of the role of miR-34a in a *Kras*-induced mouse lung cancer model demonstrated that miR-34a down-regulation alone was not sufficient to promote an oncogenic effect rather, the combination of miR-34a down-regulation and altered p53 expression strongly promotes tumourigenesis, suggesting the p53-miR-34a positive feedback loop is an important feature in tumourigenesis (Okada et al., 2014). Patients with an up-regulation of miR-34a may therefore have wild-type p53 status which could contribute to a better prognosis. However another study utilising TCGA data to investigate the role of miR-34a expression in glioblastoma also identified patients with a low expression of miR-34a as having better prognosis than those with a high expression which was further confirmed with microarray analysis (Genovese et al.,

2012). Furthermore, the study by Genovese *et. al.* demonstrated that miR-34a down-regulation was most frequently associated with the proneural glioblastoma subtype, which displays a better prognosis than other glioblastoma subtypes. The glioblastoma subtypes of the patients whose samples were used in this thesis were unknown, therefore further investigation utilising samples whose subtype is known would identify whether the miRNA biomarkers in this thesis are subtype specific.

MiR-92a was up-regulated in the tissue of glioblastoma patients in the TCGA dataset, but was not correlated with age. MiR-92a has previously been reported to be up-regulated in glioblastoma tissue (Niu et al., 2012). However, no significant difference in miR-92a expression was observed between genders in glioblastoma tissue in contrast to the gender difference observed in the serum data in Chapter 4. MiR-92a was also up-regulated in the male frozen tissue sample in Section 5.2.1, but was down-regulated in the female tissue sample. The differences in expression between the TCGA data and the data obtained in Section 5.2.1 as well as 4.2.2 may be due to differences in methods as microarray data and qRT-PCR expression data can often differ (Pritchard et al., 2012a). The small sample size of frozen tissue used in this study and the large sample size of the TCGA dataset, however, suggests that the TCGA data can be interpreted with more confidence. On the other hand, miRNA microarray data often requires validation by qRT-PCR (Morey et al., 2006). The high expression of miR-92a in glioblastoma tissue of both male and female patients suggests that the differences in serum miR-92a expression observed in Section 4.2.4 may be due to gender differences in exosomal packaging and release. Patients with a higher expression of miR-92a had a better prognosis than those with a lower expression. Similar to miR-34a TCGA survival data, miR-92a, an oncomiR which supresses apoptosis, thereby exhibiting pro-tumourigenic effects (Niu et al., 2012), therefore the better prognosis observed with an up-regulation of miR-92a does not conform to its tumourigenic role.

MiR-20a was also relatively high in the tissue of glioblastoma patients and, as observed in the serum data (Section 4.2.4), expression was inversely correlated with age. The inverse correlation with age for miR-20a has previously been reported in a number of replicative cell aging models (Hackl et al., 2010). Patients with a higher expression of miR-20a were found to have a better median survival than those with a down-

regulation as seen in the serum. A previous study utilising TCGA data also identified miR-20a as being highly expressed in glioblastoma and also to be correlated with a better prognosis (Srinivasan et al., 2011). The TCGA data in Section 5.2.2 and the serum data in Section 4.2.4 showed a similar trend in miR-20a expression and prognosis, suggesting that the profile of this miRNA in the serum closely reflects that of glioblastoma tissue.

Furthermore, analysis of correlation in the TCGA dataset in Section 5.2.2 between miR-20a and miR-92a expression in the tissue of glioblastoma patients showed that expression of these two miRNAs was significantly correlated. The correlation of expression between these two miRNAs is likely due to their location in the same cluster, the 17~92 cluster of miRNAs located within the non-protein coding gene *MIR17HG*, which is transcribed together (Hackl et al., 2010).

Analysis of miR-30c and miR-150 tissue expression in the TCGA dataset showed no significant change in expression for either miRNA in glioblastoma patients. Furthermore miR-30c and miR-150 displayed no gender or age related differences in expression and had no association with survival of glioblastoma patients. As mentioned previously miR-150-5p dysregulation has not been associated with glioblastoma to date (Yang et al., 2013a).

Analysis of tissue and serum miRNA biomarker expression of patients used in this current study was performed to determine whether tissue miRNA expression correlated with serum expression. Expression of miR-34a in the serum of 60+ glioblastoma patients was significantly higher compared to miR-34a expression in the tissue. As previous studies have identified miR-34a to be down-regulated in the tissue of glioblastoma patients (Gao et al., 2013) and serum data obtained in Section 4.2.4 showed a high expression of miR-34a in the serum of glioblastoma patients, the trend of higher serum miR-34a expression in Section 5.2.3 confirms the serum data obtained in this study and the tissue data obtained in other studies. As miRNA expression in the circulation has been found to be lower than miRNA expression in donor cells (Skog et al., 2008), the observation that miR-34a is expressed at a higher level in serum compared to glioblastoma tissue suggests that either miR-34a is actively secreted at a high level from glioblastoma or is secreted from an alternative source.

Expression of miR-92a in the serum of male glioblastoma patients was reduced compared to tissue expression. MiR-92a is known to be up-regulated in the tissue of glioblastoma patients (Niu et al., 2012), which is reflected in its high expression in the serum of male glioblastoma patients in Section 4.2.4. The lower expression of miR-92a in the serum compared to tissue is again likely to be due to the general lower expression of miRNA in the circulation compared to tissue as mentioned previously (Skog et al., 2008). Expression of miR-20a in matched serum and tissue samples was not significantly different, suggesting that expression of miR-20a in serum is similar to that in the tissue of glioblastoma patients as observed previously in the serum data Section 4.2.4 and the TCGA data in Section 5.2.2.

The localisation of the miRNA serum biomarkers in glioblastoma tissue was determined using ISH. Three of the five miRNA biomarkers were selected for investigation using ISH. ISH provides a visual representation of miRNA expression in tissue sections as qualitative data, qPCR analysis of tissue miRNA expression was also performed in parallel to the ISH study to provide quantitative data and validate the ISH data. As patient tissue samples were limited, only five matched patient tissue lysate and tissue sections were used in the ISH study meaning ISH data for a small sample of the patient cohort was compared to the qPCR data, however those analysed by both ISH and qPCR could provide some information on the correlation between the localisation of the miRNA biomarkers in the tissue sections and expression of the miRNAs in the tissue determined by qPCR. Optimisation of the probe hybridisation temperature exhibited positive signal for both miR-21 and U6 snRNA positive controls and negative staining for the scrambled probe at both temperatures. A final hybridisation temperature of 37 ° C was selected as a higher temperature reduces the extent of non-specific binding of the probes to similar sequences (Nielsen, 2012).

In Situ analysis of miR-20a in female and male glioblastoma patient tissue sections displayed both positive and negative staining for the cohort. Differences in miR-20a expression between the glioblastoma patient tissue sections is similar to that observed for serum miR-20a expression in which up-regulation of miR-20a was observed for a sub-group of the patient cohort who were also observed to have a better prognosis than those without an up-regulation in miR-20a expression. This may be due to differences in miRNA expression between different molecular subtypes of glioblastoma

(Tang et al., 2013). Comparison of tissue lysate and tissue section miR-20a expression showed similar expression of miR-20a in the tissue lysate when compared to tissue section miR-20a expression. MiR-20a ISH signal was uniform across the majority of the patient sections, exhibiting both cytoplasmic and nuclear expression. Patients exhibiting cytoplasmic and nuclear staining of miR-20a expression in the sections exhibited similar ΔCt values of miR-20a in tissue lysate and serum miR-20a expression. BTNW 816 frozen tissue miR-20a expression was observed to be lower than serum miR-20a expression. Furthermore, ISH miR-20a expression for this patient was observed to be restricted to the nucleus of cells within the section. The localisation of miR-20a in the nucleus of BTNW 816 suggests miR-20a expression is enriched in the nucleus which has been observed for a number of miRNAs previously (Roberts, 2014). The combination of both nuclear and cytoplasmic expression of miR-20a in the remaining patient sections may attribute to the higher tissue expression determined by qPCR compared to the tissue expression of BTNW 816.

In Situ analysis of miR-34a in male and female glioblastoma patient tissue sections showed a correlation between expression and age similar to that observed in the serum of glioblastoma patients in Section 4.2.8. Comparison of frozen tissue and serum miR-34a expression exhibited a lower expression of miR-34a in the tissue of glioblastoma patients compared to matched serum. This confirms previous findings of a reduced expression of miR-34a in glioblastoma tissue in other studies as well as in this thesis in Section 5.2.3 (Gao et al., 2013, Li et al., 2009). Patients with a down-regulation in frozen tissue miR-34a expression compared to serum also showed weak or negative miR-34a signal in the tissue sections, showing a correlation between ISH and qPCR miR-34a expression. BTNW 850 a glioblastoma patient aged between 20 and 39 years exhibited a positive miR-34a signal in the tissue section, this patient glioblastoma may exhibit wild-type p53 status, which targets miR-34a and promotes its expression in the tissue of glioblastoma patients (Okada et al., 2014), which would therefore account for the positive signal observed.

In Situ analysis of miR-92a in female glioblastoma patients exhibited no staining for all sections excluding BTNW 743 and BTNW 1186. ISH and qPCR data for BTNW 429 showed contrasting results, ISH for this patient showed a negative signal for miR-92a in the tissue section however miR-92a tissue expression determined by qPCR exhibited

an up-regulation in miR-92a expression compared to the matched serum sample. This may be due to the section utilised for ISH being taken from around the glioblastoma, thereby containing non-cancerous cells with a different miR-92a expression. MiR-92a expression in tissue sections of male glioblastoma tissue sections showed positive staining for all but one of the patients, suggesting that miR-92a is up-regulated in the tissue of male glioblastoma patients. MiR-92a has previously been found to be up-regulated in the tissue of glioblastoma patients (Niu et al., 2012) as well as in the serum of male glioblastoma patients shown in Section 4.2.4. Both male and female patients with a positive miR-92a ISH signal displayed a down-regulation of tissue miR-92a expression compared to matched serum by qPCR, this suggests patients with tissue miR-92a expression have a higher expression of miR-92a in the serum.

ISH analysis of miR-92a and in particular miR-20a localisation displayed strong nuclear expression. All three miRNAs showed both nuclear and cytoplasmic signal in the tissue sections of patients, previous analysis of miRNA localisation intracellularly has found that the majority of mature miRNAs are distributed in both nuclear and cytoplasmic compartments (Roberts, 2014). A study of rat primary cortical neurons identified a significant enrichment of mature miR-92a in the nucleus (Khudayberdiev et al., 2013), as observed in Section 5.2.3.

Recently, the presence of miRNAs in the cerebrospinal fluid of glioblastoma patients has been reported (Teplyuk et al., 2012, Baraniskin et al., 2012). The presence of the BBB, a highly selective barrier, leads to a restricted composition of cerebrospinal fluid, therefore the detection of miRNAs in this biofluid may be more specific to glioblastoma and provide a more accurate biomarker profile of the tumour due to a lower level of miRNA released from other non-cancerous cell types. The miRNA profile of cerebrospinal fluid was investigated as a comparison to serum as a source of biomarkers. Total RNA obtained from cerebrospinal fluid samples following extraction was markedly lower than that of serum (Appendix 5). Following identification and validation of miRNA biomarkers in the serum, these markers were measured in cerebrospinal fluid. MiR-30c, miR-34a and miR-150 were not detected in the cerebrospinal fluid of glioblastoma patients however miR-92a and 20a were found to be expressed. Due to the limited availability of cerebrospinal fluid samples, no samples from the 20-39 age group were analysed for this study, however expression of miR-20a

was up-regulated in the cerebrospinal fluid of glioblastoma patients aged between 40 and 59 years compared to those aged over 60 years and patients without glioblastoma, this trend was also observed in the serum of glioblastoma patients. MiR-20a was found to be down-regulated in the cerebrospinal fluid of glioblastoma patients aged over 60 years compared to those without glioblastoma. MiR-92a was up-regulated in the cerebrospinal fluid of male glioblastoma patients and down-regulated in the cerebrospinal fluid of female glioblastoma patients, this trend was also observed in the serum of glioblastoma patients.

The detection of two of the three serum miRNA biomarkers in the cerebrospinal fluid of glioblastoma patients suggests that this biofluid could also be used as a source of biomarkers. MiR-34a however was not detected in the cerebrospinal fluid of glioblastoma patients, which could have been due to the low yield of RNA obtained from these samples or alternatively miR-34a may not originate from the glioblastoma which correlates with previous studies (Gao et al., 2013). The use of a larger biomarker panel for glioblastoma would provide a more accurate representation of disease state, therefore serum miRNA, in this instance, would provide the best source of biomarkers. To further investigate the origin of the four serum biomarkers in the serum of glioblastoma patients, lymphocytes isolated from the buffy coat of glioblastoma patient blood samples were analysed for expression of the miRNA biomarkers. Expression of all four miRNA biomarkers was not significantly different in glioblastoma patient lymphocytes compared to control patient serum. This suggests that the change in expression in the biomarkers detected in the serum is not related to an alteration in expression in lymphocytes. The small sample size however, did not permit analysis of miRNA biomarkers in patient lymphocytes grouped by age and gender, which could have provided a more in depth analysis of miRNA expression in lymphocytes. Furthermore, the use of control lymphocytes from patients with other neoplastic brain diseases could have also affected the data obtained. Detection of the miRNA biomarkers in the serum utilised serum from control patients with no diagnosed neoplasm and the expression of the miRNA biomarkers was not investigated for specificity for glioblastoma alone. The miRNAs identified in the serum therefore, may also display a similar dysregulation in other glioma types and grades as well as other brain neoplasms. Further work utilising a larger patient cohort and control samples

obtained from non-neoplastic patients grouped by age and gender could therefore provide a more accurate description of miRNA biomarker expression in lymphocytes in glioblastoma patients.

Data from Chapter 5 have shown differences in correlation between serum and tissue miRNA biomarker expression. MiR-20a exhibited a similar expression between serum and tissue samples in the human serum cell culture model in Section 5.2.1, TCGA data in Section 5.2.2, qPCR serum and tissue expression comparison in Section 5.2.3 and from comparison of ISH data and qPCR tissue data in Section 5.2.4. Furthermore correlation between age and miR-20a expression and prognosis was observed in the TCGA dataset as previously seen in serum data in Section 4.2.4. TCGA data, qPCR and ISH analysis all identified miR-20a expression trends to be closely similar between serum and tissue samples. This suggests that the expression of miR-20a in the serum closely reflects the expression in glioblastoma tissue, therefore circulating miR-20a is likely to originate from the glioblastoma itself. Expression of miR-34a was reduced in the tissue compared to serum in Section 5.2.3 and 5.2.4 as observed in Section 4.2.4 however, TCGA expression data did not correlate with glioblastoma tissue miR-34a expression in the qPCR and ISH data. MiR-34a was not expressed in the cerebrospinal fluid of glioblastoma patients and results from lymphocyte expression were inconclusive. Taken together, the origin of miR-34a in the serum of glioblastoma patients is still unknown. MiR-92a expression was significantly higher in glioblastoma tissue in the TCGA dataset analysis, matched serum and tissue samples and ISH. The gender difference observed in Chapter 4 in the serum however, was not observed for glioblastoma tissue in this chapter. This suggests that although miR-92a may originate from the tumour, gender differences in exosomal packaging and release may have led to the higher detection observed in male glioblastoma serum samples.

6. Discussion

6.1 Summary

This thesis aimed to profile the serum miRNA expression of glioblastoma patients and identify a panel of dysregulated miRNAs which could be used for the diagnosis of glioblastoma. A human serum cell culture model was developed to investigate the effects of glioblastoma and non-cancerous human serum on miRNA expression within the glioblastoma cell line U87MG and the non-cancerous astrocyte cell line SVGp12. Following investigation of human serum effects in cell culture, profiling of serum from glioblastoma patients identified a number of dysregulated miRNAs which, following validation, led to a panel of four miRNA biomarkers which could potentially be used for diagnosis of glioblastoma. It was further demonstrated that miR-20a may also have the potential to be a prognostic biomarker, likely to be associated with the oligoneural glioblastoma subtype (Kim et al., 2011). To further understand the miRNA biomarker panel identified, studies were performed in order to elucidate the origin of these miRNAs in relation to the presence of the glioblastoma and it was demonstrated that some of the miRNAs from this panel may originate from the glioblastoma itself and other miRNAs in the panel may originate from alternative sources such as cells of the immune system. Ultimately, these studies have identified a miRNA biomarker panel which could improve or enhance current diagnostic techniques for glioblastoma as well as further our understanding of how dysregulation of miRNAs may lead to glioblastoma progression, malignancy or prognosis.

6.2 Development of a human serum cell culture model for investigation of miRNA expression

Current research into the expression and targets of miRNAs for disease *in vitro* employs immortalised human cell lines cultured in medium containing a supplement of 10 % FBS. Whilst this provides a good starting point for *in vitro* studies, the use of serum obtained from a separate species is likely to affect the data obtained from such studies. The foetal origin of FBS means it is rich in growth factors and hormones which promote the growth of cells. Although it is known that FBS contains growth promoting components, the exact composition is unknown and some components have not been identified or the effect on cell lines is unknown (Brunner et al., 2010). Batch to batch variability in FBS composition is also a limitation on studies utilising cultured cells (Witzeneder et al., 2013). The first aim of this thesis was to develop a cell culture

model using human serum from patients both with and without glioblastoma and to investigate the characteristics of U87MG and SVGp12 cells in this model.

The culture of U87MG and SVGP12 cells in human serum in Chapter 3 resulted in changes in rate of proliferation, morphology and miRNA expression. This suggested that the serum type used does have an effect on the characteristics of a cell line and subsequently the data obtained. Whilst the use of a human serum cell culture model may be more closely representative of *in vivo* conditions, the use of immortalised cell lines themselves may also influence the data obtained from *in vitro* studies. Analysis of miRNA profiles of U87MG and SVGp12 cells cultured in standard 10 % FBS conditions identified a number of miRNAs whose expression did not match that of glioblastoma tissue *in vivo*. Prolonged culture of these cell lines and the process of immortalisation introduces mutations and chromosomal aberrations that are not representative of the tissue source (Li et al., 2008), therefore whilst the human serum model provides a closer representation of glioblastoma *in vivo*, data must still be interpreted with caution and further studies must be performed using primary cell lines and tissue to validate findings.

The culture of U87MG and SVGp12 cells in the human serum model was found to alter the intrinsic cellular expression of miRNAs as well as the secreted extracellular miRNA when compared to cells cultured in FBS. The alteration of intrinsic miRNA expression was not always found to be correlated with extracellular expression and *vice versa*. This suggests that the process of miRNA packaging and release is a regulated process that is not solely dependent on the expression levels of a particular miRNA within cells. Whilst still not wholly understood, the packaging of miRNAs into exosomes and subsequent release into the extracellular environment is known to be a selective process (Hu et al., 2012). This suggests that miRNAs which are released into the circulation have a specific role extracellularly. The selective release of miRNAs from glioblastoma can alter the tumour microenvironment promoting growth, invasion and angiogenesis (Li et al., 2013). The measurement of such miRNAs, therefore, offers a good source of biomarkers which could provide information about an individual's disease for personalised treatment. It has been previously shown that FBS itself contains miRNAs of a bovine origin packaged within exosomes (Shelke et al., 2014) which could potentially share sequence similarity with the human miRNAs analysed in

Chapter 3. The use of FBS as a control therefore, from which changes in miRNA expression were measured, prevented expression analysis of both bovine and human miRNAs. To further improve the reliability of the studies performed in Chapter 3 using the human serum cell culture model exosome depleted FBS could have been used for culture (Shelke et al., 2014), thereby eliminating the presence of any miRNAs other than those present in the human patient serum. The human serum cell culture model developed in Chapter 3 therefore provides a novel *in vitro* model which more closely represents *in vivo*.

6.3 Identification and Validation of Serum MiRNA Biomarkers

The primary aim of this thesis was to identify miRNAs in the serum of glioblastoma patients which could be used as biomarkers to improve diagnosis and treatment decisions. Data from Chapter 3 indicated differences in composition between serum from glioblastoma patients and non-cancerous control patient serum. From these data, Chapter 4 aimed to elucidate whether one component of serum, miRNA, differed between glioblastoma patient and control patient serum and whether this could be used to identify miRNA biomarkers for glioblastoma. Whilst many studies have investigated the identification of circulating miRNA biomarkers, few have considered the effects of gender and age on miRNA expression. One such previous study utilised the TCGA dataset to determine race and gender specific miRNAs which could be associated with the prognosis of glioblastoma patients (Delfino et al., 2011). Delfino *et al.* identified 19 miRNAs with gender, race and therapy associations with glioblastoma survival.

This thesis, therefore, aimed to identify miRNA biomarkers within patients grouped by aged and gender, to provide biomarkers with a greater specificity initially for diagnosis but also for prognostic purposes. Selection of age groups was based on the study by Crocetti *et al.,* (2012) who grouped epidemiological data by the age groups of 20-39, 40-59 and over the age of 60 years.

To further improve the validity of the miRNA biomarkers identified in this thesis, initial identification was performed using a small patient cohort, further confirmed using a larger patient sample set and subsequently validated using an independent patient cohort the size of which was determined using power analysis. Power analysis provides

a confidence level that statistically significant differences in expression of miRNA between groups will be detected by the study if one exists, and ensures the sample number is large enough to detect such a difference (Suresh and Chandrashekara, 2012). In this study, the expected difference, also known as effect size (Suresh and Chandrashekara, 2012), was a fold change of one for each miRNA, therefore the power analysis determined the sample size required to detect a difference of one fold change between the glioblastoma and control patient cohorts. The minimum power required for a study is 80 % power (Suresh and Chandrashekara, 2012), therefore the appropriate sample size for each miRNA was selected to provide 80 % power. For miRNAs which required a small sample number for 80 % power, more samples were included to increase the power to 90 % and thereby increase confidence in statistical significance, therefore validation of miR-34a and miR-20a provided 90 % power.

Initial miRNA identification was performed using the miScript brain cancer array panel containing 84 miRNAs with a known role in brain cancers, including glioma. Whilst this provided a large number of miRNAs as a starting pool for biomarker identification, the use of this array did not permit the identification of novel miRNAs with unknown roles in brain cancer. Further studies using alternative methods such as the employment of cDNA libraries from size fractionated small RNA (Berezikov et al., 2006) or microarrays (Liu et al., 2008) could be utilised to identify novel miRNAs in the serum of glioblastoma patients and expand the biomarker panel. Although the miScript brain cancer panel allowed profiling of a large set of miRNAs, only one sample could be analysed per 96 well plate hence due to the cost of the miScript panels, only a cohort of three glioblastoma patients per group with a limited number of control patients could be analysed using this method. Further analysis of dysregulated miRNAs in a larger patient cohort utilised the NCode first strand cDNA synthesis kit and goTaq SYBR green master mix which provided a lower cost method for miRNA expression analysis and therefore permitted analysis of larger patient cohorts. The miScript and NCode methods use the same principal for the reverse transcription and qRT-PCR of miRNAs. Prior to reverse transcription, poly(A) polymerase was used to add a poly(A) tail onto the miRNA (Benes and Castoldi, 2010). A universal reverse primer and a forward primer specific to the target miRNA was subsequently used for qPCR (Benes and Castoldi, 2010). Both miScript and NCode use the SYBR green detection method to

perform relative quantification and the cel-miR-39 spike in was used in both instances for normalisation (Kroh et al., 2010). Currently there is no universally agreed endogenous control for circulating miRNA qRT-PCR normalization. Analysis of tissue miRNA expression normally utilises the U6 snRNA endogenous control. A number of controls have been proposed and are utilised for circulating miRNA expression studies however, other studies investigating the nature of expression of some of these endogenous controls have found altered expression in diseased states. To effectively normalise qPCR data for miRNA expression in this thesis, the Cel-miR-39 spike in was utilised. With no mammalian homologue, the use of the cel-miR-39 spike in permitted confidence in no *in vivo* related variability and could be additionally used to determine efficiency of the RNA extraction process (Kroh et al., 2010). Maintaining the same methods for detection, relative quantification and normalisation of miRNA ensured minimal bias between data generated from the profiling and validation studies.

The primary method of miRNA detection utilised in this thesis was qRT-PCR, quatification of miRNAs using this method presents some unique challenges compared to the quantification of mRNA. The small size of miRNAs and GC content provides challenges in primer design, requiring primers which include the whole mature miRNA sequence to obtain an appropriate Tm between 55 °C and 65 °C (Benes and Castoldi, 2010).

MiRNAs which are to be used as biomarkers must have little variation in expression between patients to ensure good sensitivity and specificity to prevent false-positive or false-negative results which could subsequently delay correct diagnosis or affect the choice of treatment strategies (Diamandis, 2010). Following serum profiling using the initial miScript miRNA panel, changes in expression in glioblastoma patient serum were analysed for significance to identify potential biomarkers for validation studies. In the patient cohort used for identification of biomarkers a number of the non-significant miRNAs were observed to vary in fold change expression between individual glioblastoma patient samples. As certain miRNAs were not significantly different from the control patient serum as a result of high variation, they were therefore deemed unsuitable for use as biomarkers. From the initial panel, there were however three miRNAs found to be significantly different in glioblastoma patient serum compared to control patient serum. MiR-34a was found to be up-regulated in the serum of

glioblastoma patients over the age of 60 years. MiR-92a was found to be up-regulated in the serum of male glioblastoma patients and miR-20a was found to be up-regulated in a subset of glioblastoma patients however further studies would need to be performed to confirm this finding. The identification of age and gender specific miRNA dysregulation in this study further supports the use of matched samples and patient grouping by age to identify specific miRNAs for different patient cohorts, which without grouping could be identified as not significantly dysregulated.

MiR-34a was found to be up-regulated in the serum of glioblastoma patients over the age of 60 years compared to age matched controls. MiR-34a expression has previously been shown to be correlated with age (Li et al., 2011b, Sawada et al., 2014). As the fold change difference of this miRNA was determined using age matched control samples, this indicated that the increase of miR-34a expression was not solely due to the age of the patients but also due to the presence of the glioblastoma. MiRNA-34a is a transcriptional target of p53 which, upon expression, mediates a number of downstream effects including cell cycle arrest and apoptosis by post-transcriptional mRNA targeting in a context-dependent manner (Table 6.1) (Okada et al., 2014). Expression of miR-34a and p53 is part of a positive feedback loop in which miR-34a targets Mdm4, a negative regulator of p53, promoting p53 activity and thereby expression of miR-34a (Okada et al., 2014).

Table 6.1 Targets of miR-34a and subsequent effects.

Target	Effect	Reference
CDK4/6	Cell cycle arrest	(He et al., 2007)
Cyclin D1/E2	Cell cycle arrest	(He et al., 2007, Sun et al., 2008)
c-Met	Cell cycle arrest	(He et al., 2007)
Myc	Apoptosis	(Wei et al., 2008)
Bcl-2	Apoptosis	(Sun et al., 2008)
YY1	Migration and proliferation.	(Yin et al., 2013)
Notch-1/Notch-2	Proliferation	(Li et al., 2009, Li et al., 2011a)
PDGFRA	Tumourigenesis	(Genovese et al., 2012)
Smad4	Tumourigenesis	(Genovese et al., 2012)

MiR-92a expression was higher in the serum of male glioblastoma patients compared to gender matched controls. MiR-92a is a protumourigenic miRNA, known to be up-regulated in glioblastoma tissue and which targets the proapoptotic Bcl-2-like protein 11 (BIM), thereby exhibiting anti-apoptotic effects (Niu et al., 2012). Furthermore, overexpression of miR-92a has been linked to angiogenesis. MiR-92a is part of the 17~92 cluster of miRNAs which produces a single primary transcript containing all six miRNAs which make up the cluster (Olive et al., 2010). The 17~92 cluster is a direct transcriptional target of c-Myc, N-Myc, E2F1 and E2F3 (Olive et al., 2010). Also known as 'oncomiR 1', the 17~92 cluster is well studied and its role in a number of cancer types has been previously identified, however the roles and targets of the specific miRNAs which make up this cluster have yet to be elucidated fully (Olive et al., 2010). The over-expression of this cluster in gliomas is characterised by high expression levels of mature miR-17 and miR-20a, however the remaining miRNAs in this cluster are not found to be up-regulated, suggesting alternative post-transcriptional processing which have not yet been fully elucidated (Lages et al., 2011). The 17~92 cluster has a homologous cluster on the X chromosome, which contains miR-92a-2, which is identical in sequence to miR-92a-1, located in the 17~92 cluster and also targets BIM (Olive et al., 2010). As discussed in Section 4.3, X-chromosome inactivation (XCI) in females could potentially explain why the up-regulation of miR-92a was observed solely in the serum of male glioblastoma patients. Alternatively, the up-regulation of miR-92a in the serum of male glioblastoma patients could be a result of an immune response towards the presence of the glioblastoma (Ohyashiki et al., 2011), although why this immune response is gender specific is unknown. Up-regulation of the 17~92 cluster in gliomas is usually characterised by higher expression of miR-17 and miR-20a, however miR-17 was not observed to be dysregulated in glioblastoma patient serum. The increase of miR-92a expression in male glioblastoma patients observed in this study may be a result of over-expression of miR-92a-2, located in the homologous cluster on the X chromosome (Olive et al., 2010).

In this thesis, miR-20a was increased in a subset of glioblastoma patients compared to non-cancerous controls in the identification cohort but not the validation cohort. MiR-20a is another miRNA which makes up the 17~92 cluster and plays a role in cell cycle regulation and apoptosis by targeting E2F1. Although miR-20a is located in the same

cluster as miR-92a, the cluster can be grouped into four different families based on the homology of the seed sequence. MiR-92a and miR-20a do not share seed sequence homology and therefore are not grouped into the same family (Mogilyansky and Rigoutsos, 2013), which may account for differences in expression profiles, with miR-20a being up-regulated in a subset of glioblastoma patients and miR-92a observed to be up-regulated in male glioblastoma patients only.

The difference in miR-20a expression between glioblastoma patients could be a result of glioblastoma subtypes which differ in gene, protein and miRNA expression (Mischel et al., 2003, Tang et al., 2013). MiR-20a over-expression has been identified previously in the pro-neural glioblastoma subtype. Further work investigating miR-20a expression in the serum and tissue grouped by glioblastoma subtypes could elucidate differences in expression which may explain the difference in expression observed in this thesis.

Higher expression of miR-20a in the serum of glioblastoma patients was found to be correlated with better prognosis compared to those who showed no change in expression compared to matched controls. Previous studies of miR-20a in glioblastoma tissue have found that an up-regulation of miR-20a is linked to improved prognosis most likely to be related to the targets of this miRNA, E2F1 and cyclin D1 (Srinivasan et al., 2011) which have both previously been linked to improved prognosis (Liu et al., 2011, Sallinen et al., 1999). The miR-20a target E2F1, is a cell cycle regulator which controls the G_1/S checkpoint of the cell cycle and targets cell cycle, DNA replication and apoptotic genes (Esquela-Kerscher and Slack, 2006). E2F1 is also part of a positive-feedback loop with MYC, which transcriptionally targets both the 17~92 cluster and E2F1 itself. Up-regulation of MYC, therefore, promotes over-expression of E2F1 which subsequently promotes further MYC expression leading to hyperproliferation. MYC is an oncogenic protein, frequently mutated or amplified in cancer and can promote both cell proliferation and apoptosis. MYC has been observed to be over-expressed in the majority of glioblastomas (Annibali et al., 2014). The up-regulation of miR-20a and miR-17, which is also encoded by the 17~92 cluster, inhibits the translation of E2F1 mRNA thereby interrupting the positive-feedback loop between E2F1 and MYC and subsequently reducing the cell-proliferative effects of MYC (Esquela-Kerscher and Slack, 2006). A previous study by Alonso *et al.* (2005) identified E2F1 as a prognostic factor for glioblastoma patients. Analysis of 61 glioma tissue samples found those with

a lower expression of E2F1 exhibited a better survival than those with an over-expression (Alonso et al., 2005). Glioblastoma tissue samples obtained from long-term survivors, defined as surviving 2 or more years after diagnosis, also displayed a lower expression of E2F1. The combination of miR-20a up-regulation and subsequent down-regulation of E2F1 may therefore contribute to a better prognostic outlook compared to patients with no change in miR-20a expression (Alonso et al., 2005). Research has found that E2F1 has contradicting effects in different cancer types, with the ability to act as both a tumour suppressor and an oncogenic factor however, in regards to gliomas E2F1 is considered to be oncogenic (Alonso et al., 2008) and therefore the up-regulation of miR-20a and inhibition of E2F1 translation is likely to be tumour suppressive in this context.

The difference in miR-20a expression between glioblastoma patients observed in this study could be likely due to difference in patient glioblastoma subtype. Whilst this difference was detected for miR-20a, no similar trends were observed for the remaining biomarkers. Current diagnostic techniques identify certain genetic markers, EGFRvIII, IDH1 and NF1, which have been associated with particular subtypes (Verhaak et al., 2010), however identification of an individual's glioblastoma subtype is not currently standard protocol during diagnosis. The cohort utilised in this study therefore, may not include the full range of glioblastoma subtypes which could have led to subtype specific expression patterns of the remaining biomarkers not being detected in this thesis. Further work establishing a panel of glioblastoma serum samples from all glioblastoma subtypes and analysis of differences in miRNA biomarker expression between these subtypes could further improve the sensitivity of the biomarker panel. This could subsequently provide information on a patient's subtype at diagnosis therefore improving treatment strategies.

6.4 Determination of circulating miRNA biomarker origin

Whilst the four serum miRNA biomarkers validated in Section 4.2.5 provided a panel which could be utilised in the diagnosis and prediction of prognosis for glioblastoma patients, further investigation to elucidate the pathophysiological origin of secreted biomarkers provided a further understanding of the cause and effect of glioblastoma on circulating miRNA expression.

The methods utilised for determining biomarker origin included qRT-PCR, TCGA dataset analysis and *in situ* hybridisation. All three methods were used to determine miRNA expression however each method provided an independent aspect on biomarker origin. Analysis of miRNA expression in glioblastoma tissue using qRT-PCR provided matched data for the patients whose serum was utilised for biomarker identification in this thesis. The use of matched samples provided an accurate, patient specific illustration of miRNA expression both in the circulation of the patients and within the glioblastoma itself. Analysis of glioblastoma tissue miRNA expression using the TCGA dataset provided a much larger patient cohort than could have been realistically analysed in this thesis and the large sample number added statistical robustness to the findings. *In situ* analysis of matched tissue sections to patient tissue lysate permitted determination of the localisation of the individual markers within the glioblastoma which, combined with qRT-PCR data of patient tissue, provided a more in depth analysis of miRNA expression in subsets of cells found within the heterogenous glioblastoma tissue.

In situ analysis of miRNA expression in glioblastoma tissue sections permits observation of specific cell types or locations of miRNA expression within the glioblastoma which would not be so readily performed using qRT-PCR or microarray analysis due to the diffuse nature of glioblastoma. Successful *in situ* hybridisation is reliant on a number of variables which can affect signal strength, including extent of fixation of the sections and the age of the sections. It has been previously found that the length of fixation of a section can reduce signal strength and require a longer protease digestion step to provide an adequate probe signal (Thompson et al., 2007). On the other hand, prolonged protease digestion can lead to poor tissue morphology and equally reduced signal (Thompson et al., 2007). Differences in the extent of fixation may explain why signal strength for certain sections utilised in Chapter 5 varied for example for the U6 positive control, however the *in situ* data obtained may still be used to determine the localisation of the miRNAs within the tumour and the additional analysis of miRNA tissue expression using qRT-PCR provided quantitative data on miRNA expression.

Following validation of the four serum biomarkers, analysis of expression was performed in cerebrospinal fluid (CSF). Due to the close proximity of CSF to the brain

and the spinal cord, the presence of a glioma within the CNS results in the alteration of CSF composition. The abundance of certain substances alters as a result of: humoral responses (Tainsky, 2009); breakdown of structures within the CSF, such as the blood-brain barrier (BBB) (de Bont et al., 2006); or as a result of up-regulated production and secretion by the glioma cells themselves (Niclou et al., 2010). The presence of miRNAs in the cerebrospinal fluid of glioma patients has initiated studies into the potential of CSF as a source of biomarkers. Although cerebrospinal fluid is not routinely obtained from patients with glioma (Baraniskin et al., 2011), the proximity of cerebrospinal fluid to gliomas and its isolation from general circulation means it could potentially provide a more specific and accurate miRNA profile in comparison to serum (Teplyuk et al., 2012).

A number of previous studies have been performed investigating the use CSF as a miRNA biomarker source for glioblastoma (Baraniskin et al., 2012, Teplyuk et al., 2012). Four biomarkers were identified in this thesis in the serum of glioblastoma patients however only two were detected in the CSF, which suggested that for this particular panel of miRNAs, serum was the more useful biofluid for detection. Previous studies investigating miRNAs as biomarkers in CSF for glioma have also identified a limited number of miRNAs which are significantly dysregulated in the CSF of glioma patients. Baraniskin *et al.,* (2011) identified two miRNAs, miR-15b and miR-21, with altered expression in CSF and Teplyuk *et al.,* (2012) also identified two miRNAs, miR-10b and miR-21, with significant altered expression in glioma CSF. Serum as a biofluid for miRNA expression therefore, can provide a larger panel of miRNAs for a more accurate diagnosis of glioblastoma.

As CSF is part of a closed system in the CNS, with a highly selective barrier, alternative sources of miRNAs are less frequent and would provide a more specific miRNA panel which would be likely to originate from the glioblastoma itself. Alternative miRNA sources present within the serum which may be affected by the presence of the neoplasm, such as immune cells, could convey a picture of the glioblastoma, and additionally responses of other systems in the body could provide information on an individual's disease, prognosis and their response to treatment. The less invasive nature of serum collection compared to CSF collection also provides a clinical advantage for serum biomarkers over CSF. Lumbar punctures are not routine for

glioblastoma diagnosis and would therefore be an additional diagnostic test and the collection of CSF during surgery would negate the use of the biomarker panel for earlier and improved diagnosis.

One of the major limitations of determining the origin of the miRNA biomarkers in this thesis was the lack of available matched samples for analysis which therefore resulted in a limited number of samples. Whilst the data in Chapter 5 can be used to elucidate the origin of miRNA expression differences between serum and tissue, further work using a greater number of matched sample sets would provide a more robust dataset.

6.5 Theoretical Application of MiRNA Biomarkers

The identification of four serum miRNAs with altered expression in glioblastoma patients could provide a panel which for both diagnosis and prognosis of glioblastoma. Extensive further studies however would be required to validate these miRNAs as biomarkers. Theoretically, female patients over the age of 60 years would be tested for miR-34a and miR-20a up-regulation, those with an up-regulation in both of these miRNAs would be diagnosed with glioblastoma. Further analysis of miR-20a expression would determine whether these patients were predicted to have a good prognosis thereby assisting in treatment decisions. Female patients aged between 20 and 59 years would be tested for up-regulation of miR-20a, those with an up-regulation would be suspected of having glioblastoma. Those with a down-regulation or no change in miR-20a expression would require further tests to confirm a diagnosis of glioblastoma. Female patients aged between 20 and 39 years would also be tested for a down-regulation in miR-30c, which in combination with an up-regulation of miR-20a, would suggest a diagnosis of glioblastoma. The diagnosis of glioblastoma from one miRNA however may result in an incorrect diagnosis and therefore miRNA expression would need to be used in conjunction with standard diagnostic techniques.

Male patients over the age of 60 years would be tested for miR-34a and miR-92a up-regulation which would result in a diagnosis of glioblastoma. Further determination of miR-20a expression would predict the prognosis of these patients. Male patients aged between 20 and 59 years would be diagnosed based on miR-92a expression and prognosis predicted by miR-20a. Male patients aged between 20 and 39 years would also be tested for a down-regulation in miR-30c, a down-regulation of this miRNA with

an up-regulation of both miR-20a and miR-92a would suggest a diagnosis of glioblastoma.

The use of miR-20a as a prognostic indicator would improve treatment decisions for individual glioblastoma patients. Patients predicted to have a poor prognosis would require aggressive therapy such as a higher and more frequent dosage of chemotherapy and radiotherapy from the beginning of their treatment. Those with a better prognosis could be treated with therapies at a lower, less frequent dosage with less side effects initially and more aggressive treatment following progression, improving the quality of life for these patients during treatment. A recent study highlighted a link between miR-20a expression and temozolomide resistance of glioblastoma (Wei et al., 2015). Treatment of the U251 glioblastoma cell lines and xenografts with a miR-20a mimic led to temozolomide resistance (Wei et al., 2015). If a similar mechanism is present *in vivo* in glioblastoma, patients with an up-regulation of miR-20a could be treated with alternative chemotherapeutic agents such as the alkylating agent cisplatin or the VEGF monoclonal antibody, bevacizumab (Neagu et al., 2015).

Whilst these diagnostic pathways using the miRNA biomarker panel would be useful for glioblastoma patients, additional studies are still required to further validate the use of this panel as biomarkers before they could be applied in a clinical setting. It has been previously found that a number of variables can affect the expression of miRNAs in the circulation including, diet, drug treatments and the presence of other diseases (Mathers et al., 2010). Patients with glioblastoma are often prescribed steroids such as dexamethasone to treat cerebral oedema (Sur et al., 2005), and anti-epileptic drugs (van Breemen et al., 2009) in addition to chemotherapeutics. Other treatments may also affect the expression of the miRNA biomarkers in the serum of patients, therefore further studies investigating the effect of steroids and anti-epileptic drugs on miRNA expression could be performed.

For this panel to be effective as a diagnostic panel for glioblastoma, the effect of variables such as those mentioned should be investigated to ensure that expression is not significantly altered which could lead to incorrect diagnosis. Furthermore, alterations of the miRNA biomarker panel expression during the course of treatment

could also be investigated to determine whether the expression of these miRNAs alters during progression of the glioblastoma and could potentially be used to predict recurrence.

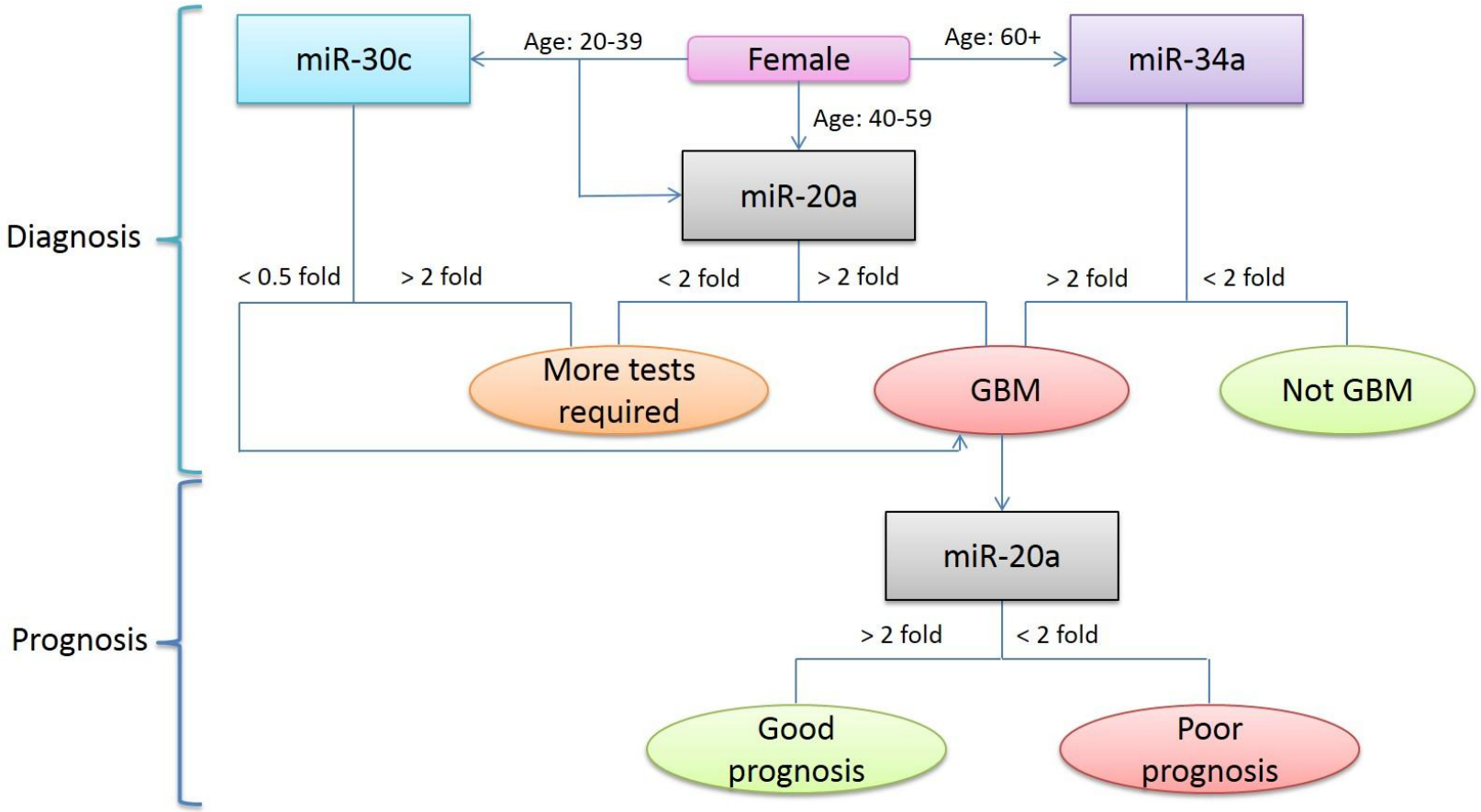


Figure 6.1 Theoretical application of the female miRNA biomarker diagnostic and prognostic pathway. Female patients between the age of 20 and 39 years suspected of having glioblastoma would be tested for miR-30c decreased expression and miR-20a increased expression. Female patients aged between 40 and 59 years would be tested for miR-20a increased expression. Female patients over the age of 60 years would be tested for miR-34a increased expression. All patients following a diagnosis of glioblastoma would be tested for miR-20a increased expression to determine prognosis.

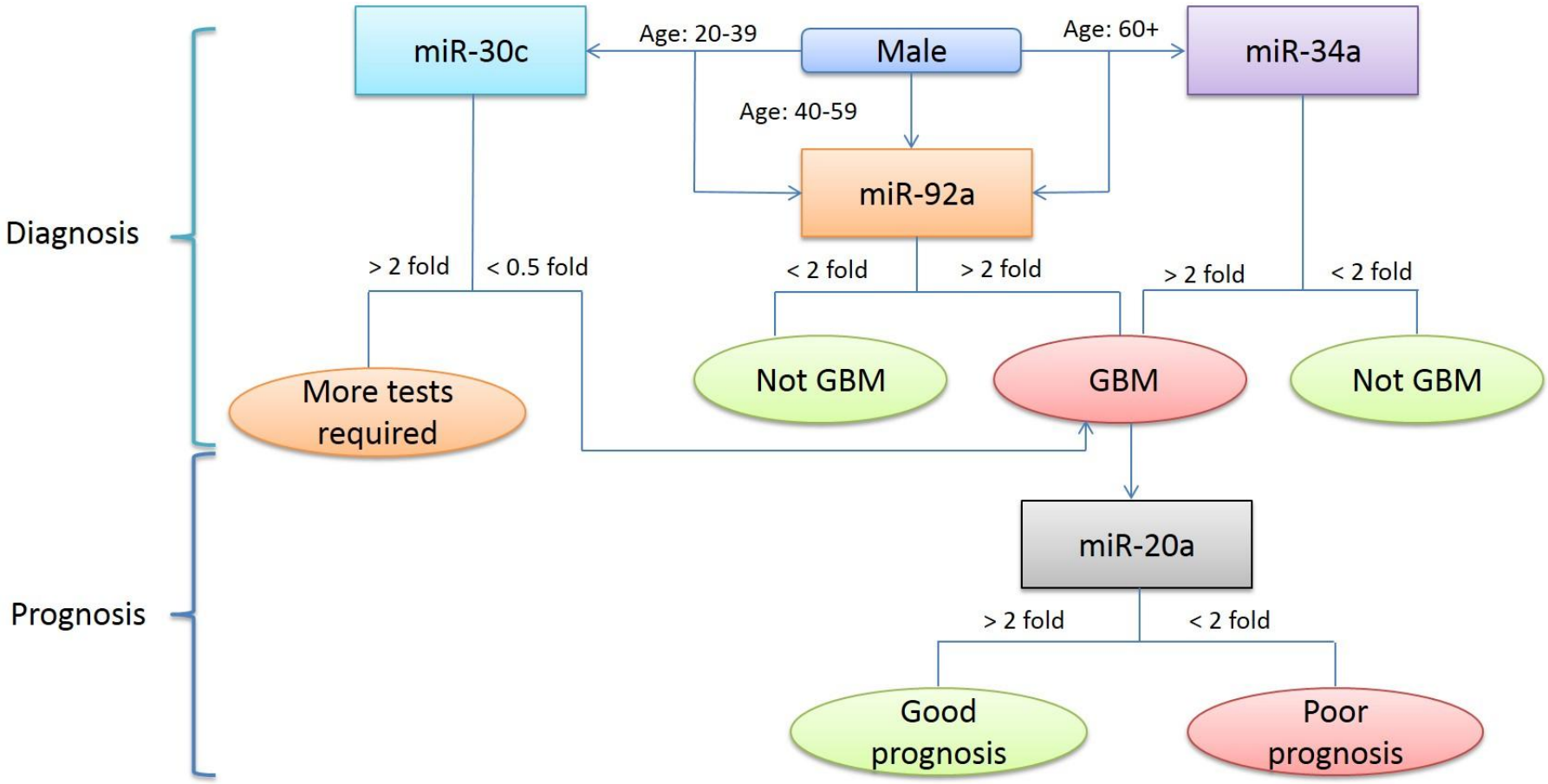


Figure 6.2 Theoretical application of the male miRNA biomarker diagnostic and prognostic pathway. All male patients suspected of having glioblastoma would be tested for miR-92a increased expression. Male patients between the age of 20 and 39 years would also be tested for decreased miR-30c expression and increased miR-20a expression. Male patients over the age of 60 years would also be tested for miR-34a increased expression. Following diagnosis all patients would be tested for miR-20a expression to determine prognosis.

Following the identification of the role of miRNAs in aberrant signalling within cancer, including glioblastoma, an area of miRNA research has begun to investigate the targeting of particular miRNAs as a novel method of treatment. The use of miRNA mimics, to restore expression of a tumour suppressor miRNAs, and miRNA inhibitors or sponges, to down-regulate an 'oncomiR' has been researched in order to develop new treatments for cancer. A miR-34a mimic has recently been advanced to Phase I clinical trials for the treatment of liver cancer in which miR-34a is down-regulated (Bouchie, 2013). The delivery of MRX34, the miR-34a mimic, restores endogenous expression levels of miR-34a, reinstating the tumour suppressor effect of this miRNA in the p53 pathway (Bouchie, 2013). The miRNA biomarkers identified in this thesis could be further studied as targets for the treatment of glioblastoma, miR-30c, up-regulated in TRAIL-resistant glioblastomas, could be targeted using a miRNA inhibitor to down-regulate expression and in conjunction with TRAIL therapy could reduce resistance of the tumour to this treatment method.

6.6 Limitations and Future Work

The primary aim of this thesis was to identify miRNA biomarkers for glioblastoma. To further improve the specificity of any miRNA biomarkers discovered in this study, patients were grouped by both age and gender. Although this improved the specificity of miRNA biomarkers, the number of variables accounted for, both genders and three age groups, restricted the number of samples studied for each group. Limiting sample number in each group therefore could have resulted in false positives or false negatives, and a smaller number of significant miRNAs which could have been identified. Further work investigating either age or gender separately would increase the sample size in each group and therefore the potential for the identification of a larger number of biomarkers.

Utilising patient samples also provided a limitation for this work in respect to sample availability. Due to the incidence of GBM being highest in patients over the age of 60 years; the number of samples from patients aged between 20 and 39 years was limited. This meant that analysis of miRNA expression in this age group was limited. In addition to this, the availability of matched tissue and serum samples also restricted the comparison of circulating and tissue miRNA expression resulting in data which

should be interpreted with caution. Future work utilising larger patient cohorts could provide more robust data on the miRNA biomarkers identified in this thesis.

Section 5.2.1 aimed to identify the effect of human serum on cell line miRNA biomarker expression. Although this provided insight into the effect of serum components on miRNA expression, it could not definitively be concluded that the miRNAs within the serum itself had cause this effect. Future work transfecting miRNA sponges and mimics could identify whether the miRNA biomarkers within human serum could affect their own expression intracellularly.

In Section 5.2.6, the expression of serum miRNA biomarkers was measured in CSF samples from glioblastoma patients. This provided insight into the differences in expression of these particular miRNA in different sample types. To determine whether CSF could be utilised as a source of biomarkers for diagnosis and prognosis however, further work could be carried utilising the 84 miRNA panel to identify CSF miRNA biomarkers that may be different to those in the serum.

Differences in miRNA expression between GBM patients such as miR-20a expression was theorised to be as a result of GBM subtypes. To further validate this hypothesis future work utilising GBM samples grouped by subtypes could be performed to identify sub-type specific miRNA biomarkers.

6.7 Conclusions

Since the discovery of tumour derived exosomes containing miRNA in the circulation of patients, the identification of miRNA biomarkers for the diagnosis and prognosis of a range of cancer types has been of great interest for the improvement of current diagnostic techniques and subsequently overall treatment of the disease. The primary aim of this thesis was to identify a panel of gender and age specific circulatory miRNAs within the serum for the diagnosis of glioblastoma and to predict prognosis. Four miRNA biomarkers were identified and validated for the diagnosis of glioblastoma, with one miRNA, miR-20a, showing significant prognostic potential.

Additional to the primary aim, a human serum cell line model was developed to provide an improved *in vitro* method for investigation of miRNA expression. This model was utilised in further studies to determine the origin of the serum miRNA biomarkers

in addition to analysis of TCGA data and *in situ* hybridisation. Future studies utilising short-term primary glioblastoma cell lines could further improve the human serum cell culture model and the representation of an *in vivo* system.

Further to the identification of four serum miRNA biomarkers, investigation into the source of the biomarkers suggested that not all the miRNAs in the panel originate from the glioblastoma itself. As well as glioblastoma tissue as a source of serum miRNAs, lymphocytes were also investigated as a source. Although an initial study was performed, the small sample size did not permit robust data to conclude whether cells of the immune system secrete the miRNA biomarkers. Further work utilising a larger sample set would provide more information on miRNA expression in lymphocytes. The investigation of alternative sources of miRNA biomarkers provides a greater understanding of the underlying pathophysiology of glioblastoma and could highlight novel treatment targets.

The serum miRNA biomarker panel identified in this thesis has the potential to provide a relatively non-invasive diagnostic test for glioblastoma. Further to the diagnostic ability of this panel, miR-20a provides a prognostic indicator. Future work elucidating difference in miRNA biomarker expression in glioblastoma subtypes could also provide a more detailed picture of an individual's glioblastoma, including likely mutations and likelihood of recurrence. This would further improve treatment strategies and the management of a patient's neoplasm which could potentially improve median survival times and patient outcome.

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Appendix 1: BTNW Tissue Bank and University Ethical Approval Documents

8 January 2013

Lisa Shaw / Charlotte Bellamy

School of Pharmacy & Biomedical Sciences

University of Central Lancashire

Dear Lisa / Charlotte

Re: STEM Ethics Committee Application
Unique reference Number: STEM 041
The STEM ethics committee has granted approval of your proposal application '**Identification of Biomarkers for Glioma Progression**'.

Please note that approval is granted up to the end of project date or for 5 years, whichever is the longer. This is on the assumption that the project does not significantly change in which case, you should check whether further ethical clearance is required.

We shall e-mail you a copy of the end-of-project report form to complete within a month of the anticipated date of project completion you specified on your application form. This should be completed, within 3 months, to complete the ethics governance procedures or, alternatively, an amended end-of-project date forwarded to roffice@uclan.ac.uk together with reason for the extension.

Please also note that it is the responsibility of the applicant to ensure that the ethics committee that has already approved this application is either run under the auspices of the National Research Ethics Service or is a fully constituted ethics committee, including at least one member independent of the organisation or professional group.

Yours sincerely

Tal Simmons

Chair

STEM Ethics Committee

Application for clinical samples/data from the

Brain Tumour North West and

the Walton Research Tissue Banks

Applicant: Duration of Project:

Name …Charlotte Bellamy……

Address …MB30 Maudland Building……

…School of Pharmacy and Biomedical Science

University of Central Lancashire

PR1 2HE

Tel: ……

Email: …cbellamy@uclan.ac.uk……

Student *(delete as appropriate)*

Project Title: ……Identification of Biomarkers for Glioma Progression……

Is the application for samples to support the project being made to:

Joint application to both banks (*delete as required*)

Funding:

Is the funding for the project

b) internal (eg funded from a researchers laboratory or institutional budget)

If the project has been submitted as a grant application for external funding?

Was the application successful? yes / no

Was the project externally peer-reviewed? yes / no

External Funding body: ……

Details of Funding: Total £ …3000…… (staff ……and consumables £3000……)

Approvals:

Ethics Approval

Does the project have ethics approval? Internal applicant applying for generic ethical approval by proxy

If yes, please supply reference number ……..and date of approval ……..

Both Tissue Banks have generic ethical approval to supply tissue/data for projects conducted by internal applicants, without the need for further ethics approval. External researchers wishing to use tissue or data supplied by the bank need to apply to REC for individual ethical approval.

Research Governance

a) Are the applicants employed by NHS establishment(s)? no

b) Does the project involve research activity using the anonymised samples/data in an NHS establishment? no

If yes to a or b, the project may require research governance approval and applicants should consult the Research Governanc Manager at their hospital

Co-applicants: Name Affiliation

Outline of Project

Please give a brief outline of the project under the headings below (not more than 1-3 A4 pages) or attach the external funding application

Hypothesis and Study Aim

Working Hypothesis: Can changes in the composition of cerebrospinal fluid, blood or serum be used to form a panel of biomarkers for the prediction of glioma progression.

(i). Main aim: The main aim of this study is to identify and isolate components of cerebrospinal fluid, blood and serum, from adult and paediatric glioma patients which are related to aspects of tumour progression, prognosis, diagnosis, likelihood of recurrence, and response to therapy. In addition, tissue samples will be utilised to compare the differences in components between the sample types.

(ii). Specific aims:

1. To identify potential components of each fluid type that could be used as biomarkers through a literature search.

2. To optimise the growth of glioma cell lines in serum by identifying the optimum concentration of serum to be used and comparing the growth affects of human serum and bovine serum by using cell culture methods.

3. To compare differences in phenotype between glioma cell lines grown in human serum and bovine serum using immunocytochemistry.

4. To isolate and quantify the abundance of specific microRNA/proteins, from glioma cerebrospinal fluid, blood, serum and tissue samples, identified from the literature search to form a biomarker panel.

5. To perform intervention studies altering the abundance of the identified biomarkers to confirm an effect on cell phenotype and behaviour.

6. To analyse the data statistically and present them as a PhD thesis.

Plan of Project

(number of cases including samples from other banks)

Proposed programme of work

(a) In vitro cell culture

1. **Basic Cell Culture**
   One glioma cell line will be used for this work, U87MG grade IV glioblastoma cell line. One non-cancerous SVGP12 astrocyte cell line will also be used. Growth curves will be made for each cell line and characterisation of the cells will be performed to confirm they are the appropriate cells.

2. **Weaning of cells off bovine serum:**
   Human obtained from adult glioma patients and patients without glioma will be used for optimisation. Glioma cell lines grown in medium supplemented with bovine serum will be weaned off the serum by decreasing the concentration of bovine serum and replacing it with equal amounts of human serum, gradually increasing the concentration of human serum to replace the full concentration of bovine serum.

3. **Culture of cells in human serum:**
   Glioma and non-cancerous cell lines will be cultured in human serum, growth curves will be performed to compare the rate of growth to cell lines cultured in normal cell culture conditions (10% foetal bovine serum (FBS)).

4. **Comparison of cell culture in different serum conditions:**
   Glioma and non-cancerous cell lines will be weaned off FBS and onto human serum.
   The cells will be incubated with the different serum and then characterised using immunocytochemistry. Changes in cell expression and phenotype will be investigated using fluorescent markers and confocal microscopy.

5. **Comparison of cell culture with different human serum samples:**
   Cells will be cultured with different human serum samples from patients with varying age and gender and immunocytochemistry will be used to determine whether the characteristics of the sample affect the phenotype of the cell.

(b) MicroRNA and Protein Isolation and Quantification

1. **Pilot studies of microRNA/protein extraction and quantification:**
   Pilot studies for method development will be performed to determine the optimum technique for the isolation, extraction and quantification of microRNAs and proteins. Method development will make use of methods used in related literature and practical development using PCR and western blotting. A single microRNA/protein will be chosen to focus on method development.

2. **MicroRNA/Protein Isolation and quantification of abundance:**
   Using the method developed, specific microRNAs and proteins will be isolated and quantified from a range of serum/whole blood/tissue/cerebrospinal fluid samples to determine differences between age, gender, cancerous and non-cancerous states. The results

will be analysed and used to form a panel of biomarkers that could potentially be used to gain information about glioma diagnosis and prognosis.

(c) Intervention Studies

1. **Alteration in the abundance of biomarker panel:**
   Cell lines will be incubated with patient samples and subsequently each potential biomarker will be isolated and removed from the sample to determine any changes in phenotype and cell characteristics using immunocytochemistry and confocal microscopy.

Experimental Methodology for A1 and A2 only (see proposed programme of study above)

Serum Replacement Study

This study will investigate the effect of different serum conditions on the microRNA expression of SVGP12 and U87MG cell lines. Studies have shown that cells in culture secrete lipoprotein microvesicles containing microRNAs which can be isolated from the medium and measured using qRT-PCR. U87MG and SVGP12 cell lines will be cultured in T25 flasks and weaned off the standard cell culture serum (10% foetal bovine serum) and onto either, non-cancerous human serum (sigma), human serum from glioma patients or serum free medium. The glioma serum will be obtained from three glioma patients and pooled for use.

Protocol

Medium was aspirated from the T25 flask and washed with PBS.

1ml of trypsin was added and incubated for five minutes.

1ml of medium containing new serum concentration was added and 1ml of cell suspension was removed.

The cell suspension was added to fresh T25 flask and 4ml of medium containing new serum concentration was also added.

The cells were incubated for 48 hours and then passaged, increasing concentration each time. The concentration of serum was increased in intervals which were: 0%, 3%, 5%, 7%, 10%, of total medium volume for the human serum and glioma serum and 25%, 50%, 75% and 100% serum free medium, as shown in Table 1.

After 72 hour incubation with the final concentration, 1ml of spent medium was collected and centrifuged to remove cells.

Total RNA including microRNAs were isolated from the media, reverse transcribed and abundance measured using qRT-PCR, with each serum condition being run in duplicate.

Table 1. Human Non-Cancerous Serum and Human Glioma Serum

Replacement Serum Concentration	Composition
0%	600 µl FBS, 5400 µl medium
3%	180µl human serum, 420µl FBS, 5400µl medium
5%	300 µl human serum, 300 µl FBS, 5400 µl medium
7%	420 µl human serum, 180 µl FBS, 5400 µl medium
10%	600 µl human serum, 5400 µl medium

Serum Free Medium

Serum Free Medium Concentration	Composition
0%	6000µl medium supplemented with 10% FBS
25%	1500µl serum free medium, 4500µl medium with 10% FBS
50%	3000µl serum free medium, 3000µl medium supplemented with 10% FBS
75%	4500µl serum free medium, 1500µl medium supplemented with 10% FBS
100%	6000µl serum free medium

Tissue Required – *Please indicate whether you require paraffin embedded tissue, fresh frozen tissue, cellular component of blood, plasma or serum and how many samples you require.*

Please indicate if all samples are required at the start of the project or if further applications for samples will be made in the light of initial findings.

Tissue, serum, cerebrospinal fluid and whole blood needed throughout the project.

For further information please contact:

BTNW Tissue Bank: Prof T Dawson email: Timothy.Dawson@lthtr.nhs.uk

Walton Research Tissue Bank: Dr C Walker email: carol.walker@thewaltoncentre.nhs.uk

Please email completed applications to:

BTNW Tissue Bank: Prof T Dawson email: Timothy.Dawson@lthtr.nhs.uk

Walton Research Tissue Bank: Dr C Walker email: carol.walker@thewaltoncentre.nhs.uk

Or to both for joint applications to both banks

For BTNW or WRTB use only:

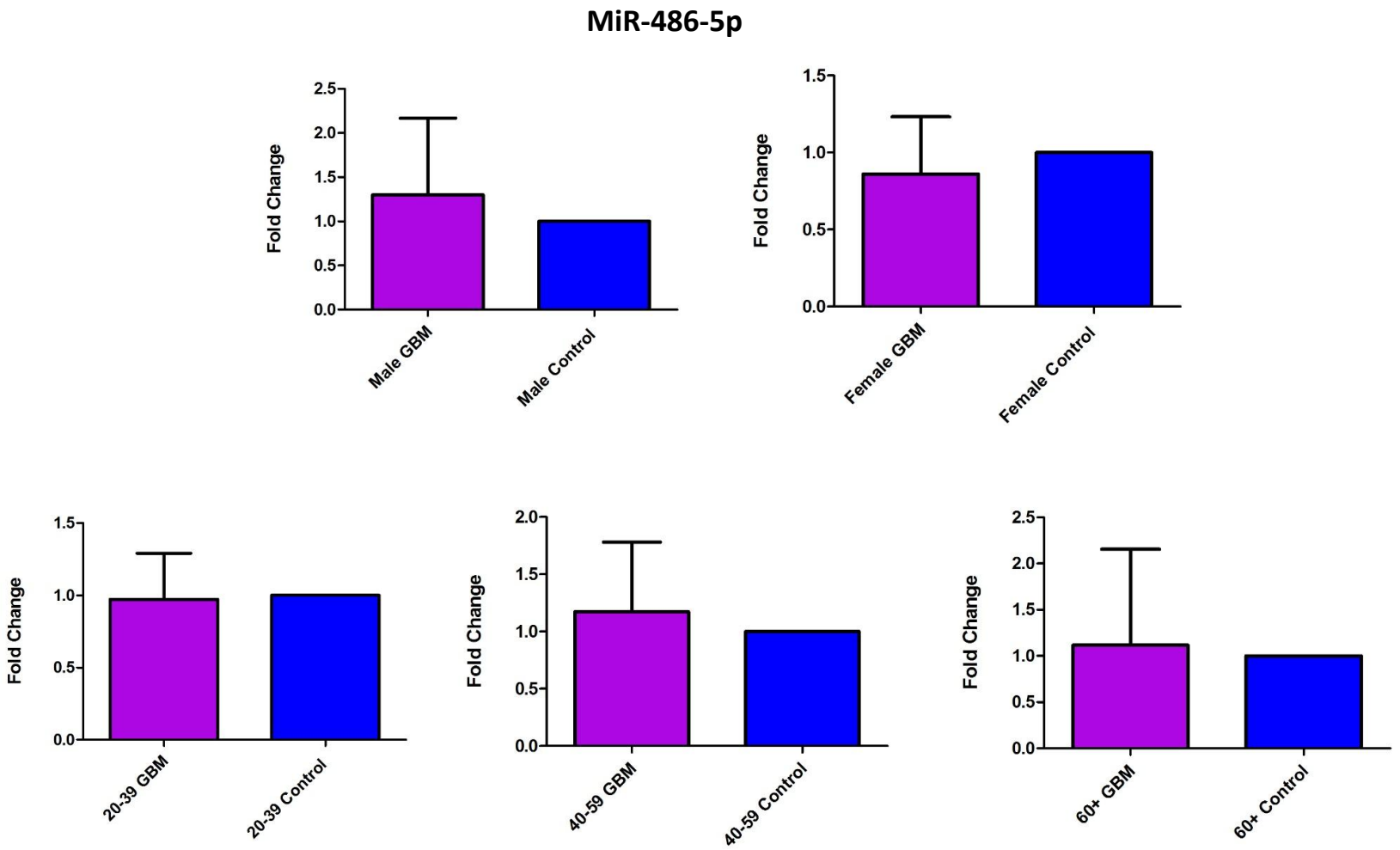
Date Application Received	08/06/2012
Application Number	1206
Project Title	Identification of Biomarkers for Glioma Progression
Date sent to BTNW/WRTB Review Panel	18/06/12
Names of Reviewers	Prof C H G Davis, Dr Tracy Warr, Prof R W Lee

Decision of BTNW/WRTB Committees	Approve Date: 19/06/2012

Appendix 2: Determination of nanodrop accuracy in measuring Total RNA samples.

RNA Standard Concentration (ng/µl)	Nanodrop Concentration (ng/µl)		
	1	2	3
1000	1008.3	1004.3	1009.7
100	102.7	103.5	102.9
10	9.7	10.0	10.0
5	4.8	4.9	4.9
1	-0.2	0.1	-0.3
0.1	-0.5	-0.9	-1.0

Appendix 3: Non-significant biomarkers from identification panel



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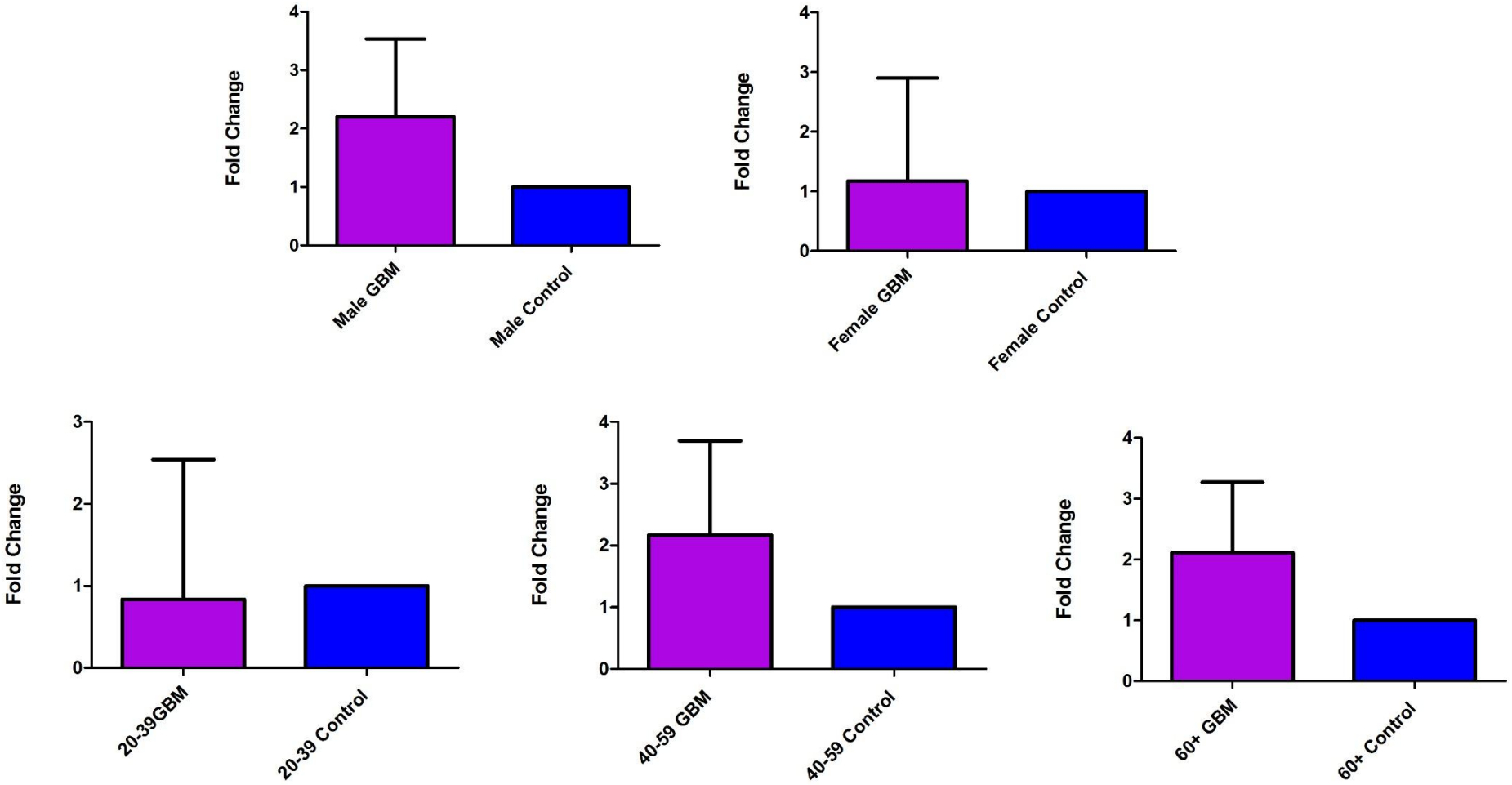
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60+ Control

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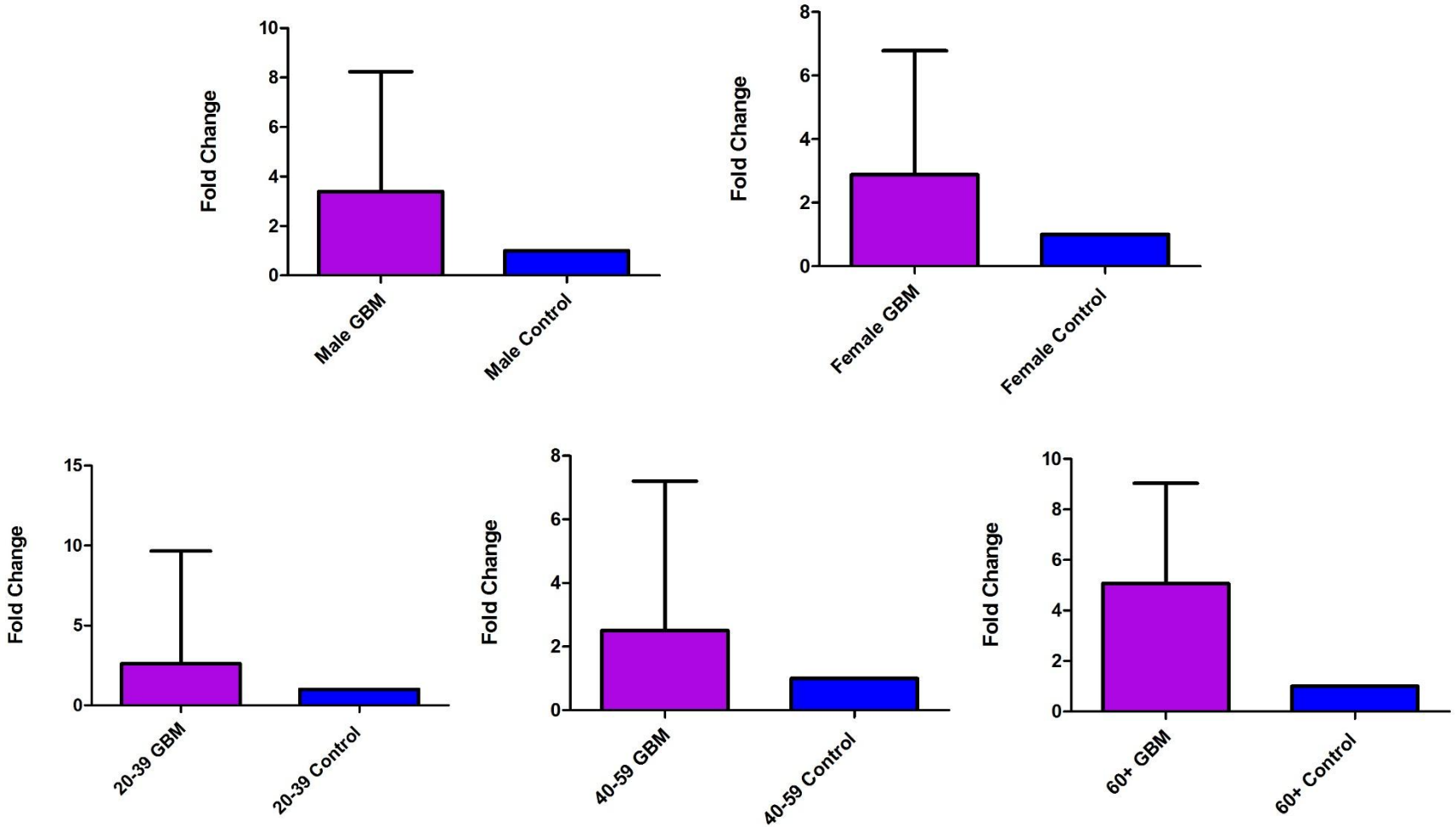
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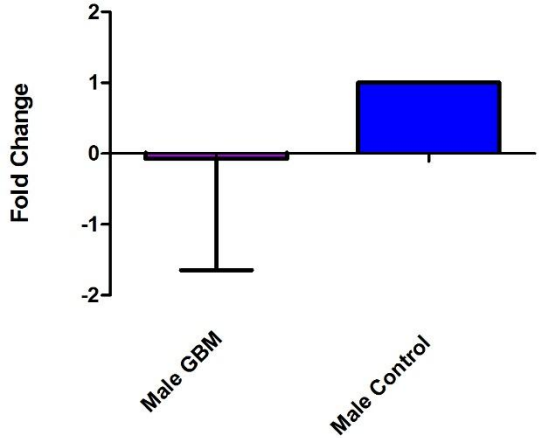
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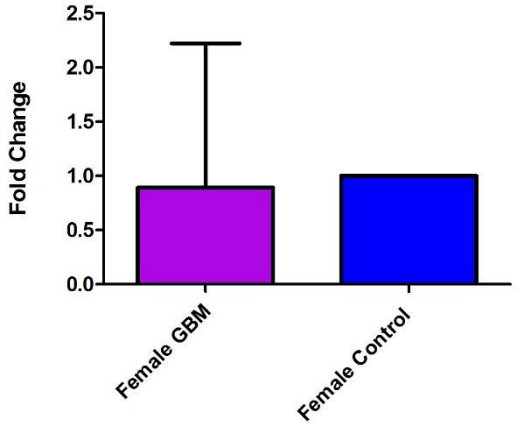
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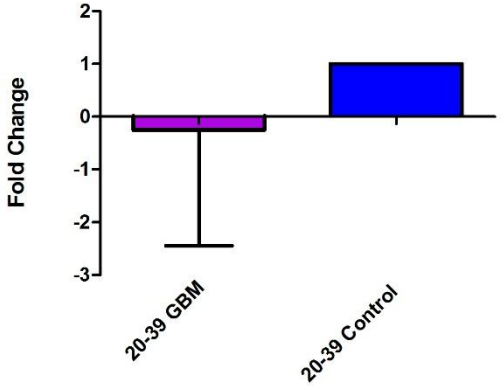
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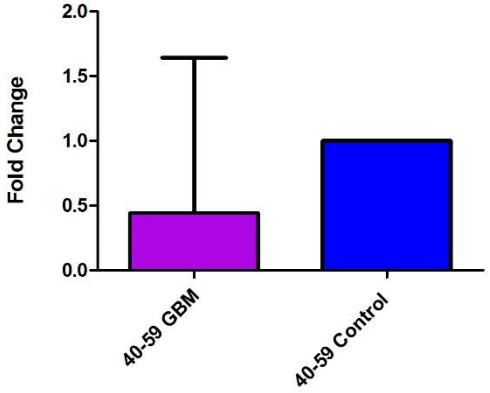
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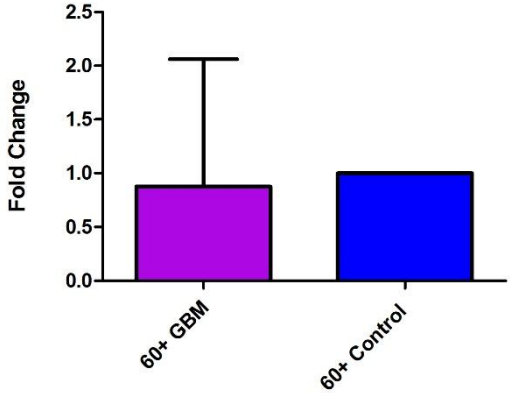
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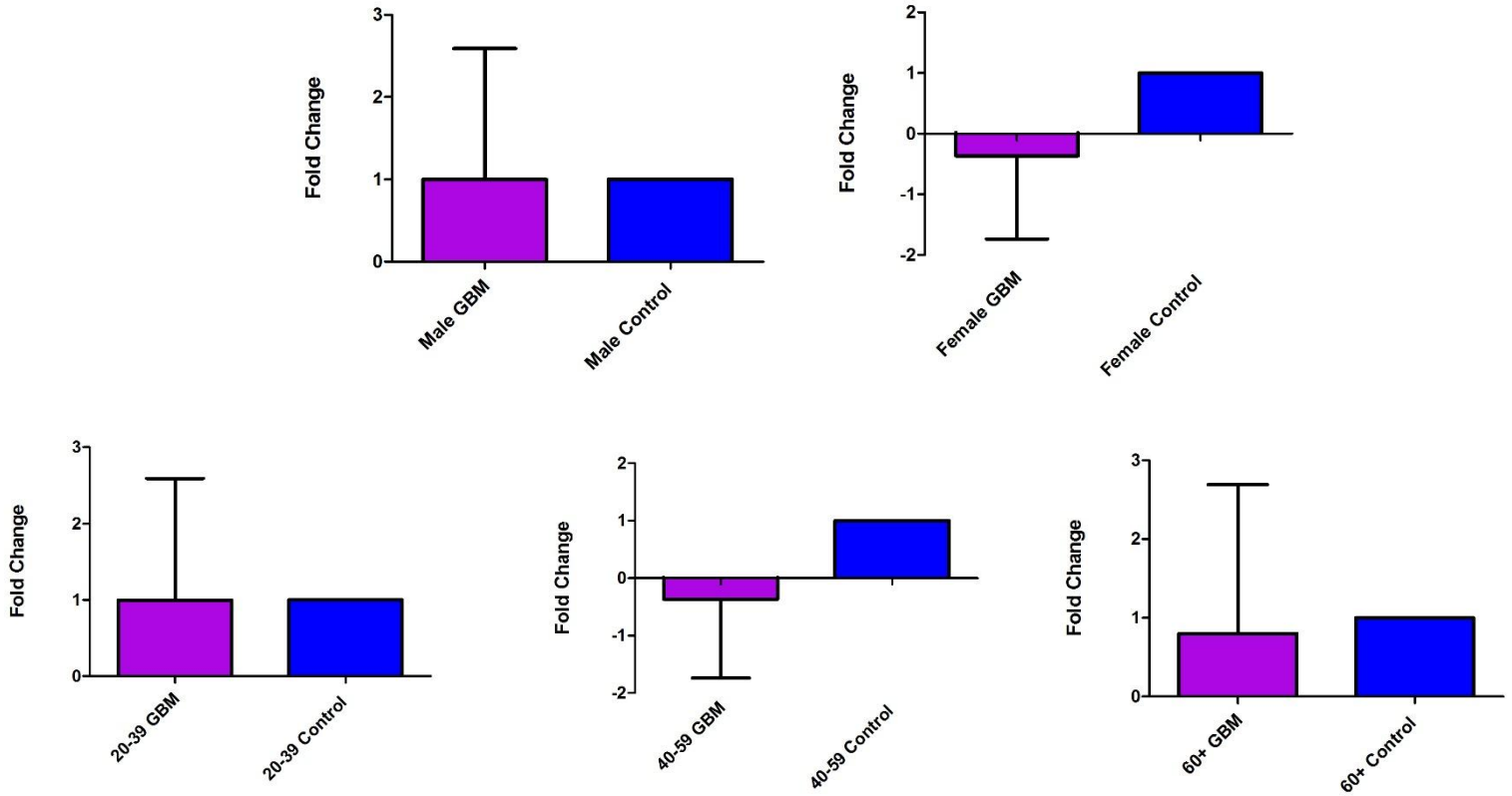
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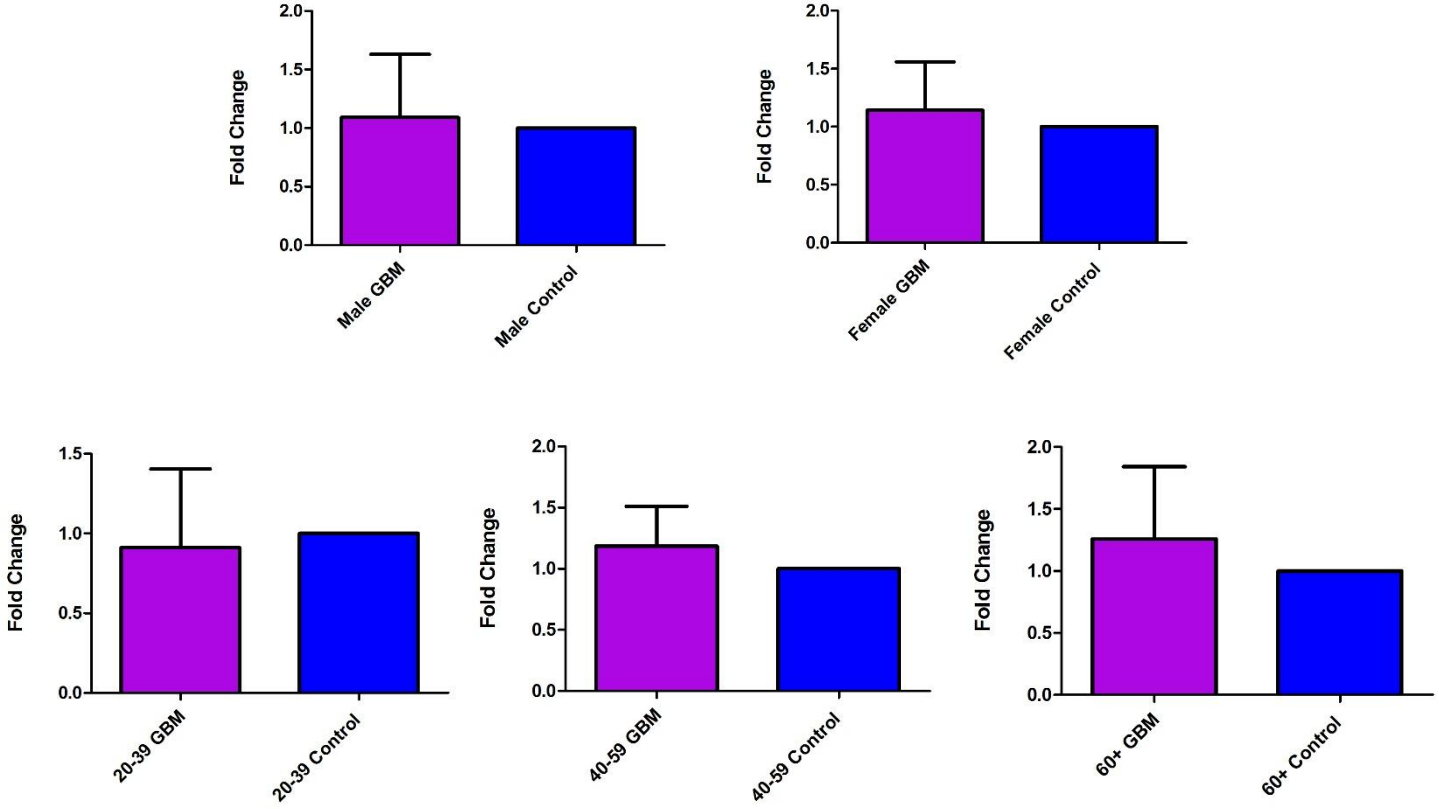
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60+ Control

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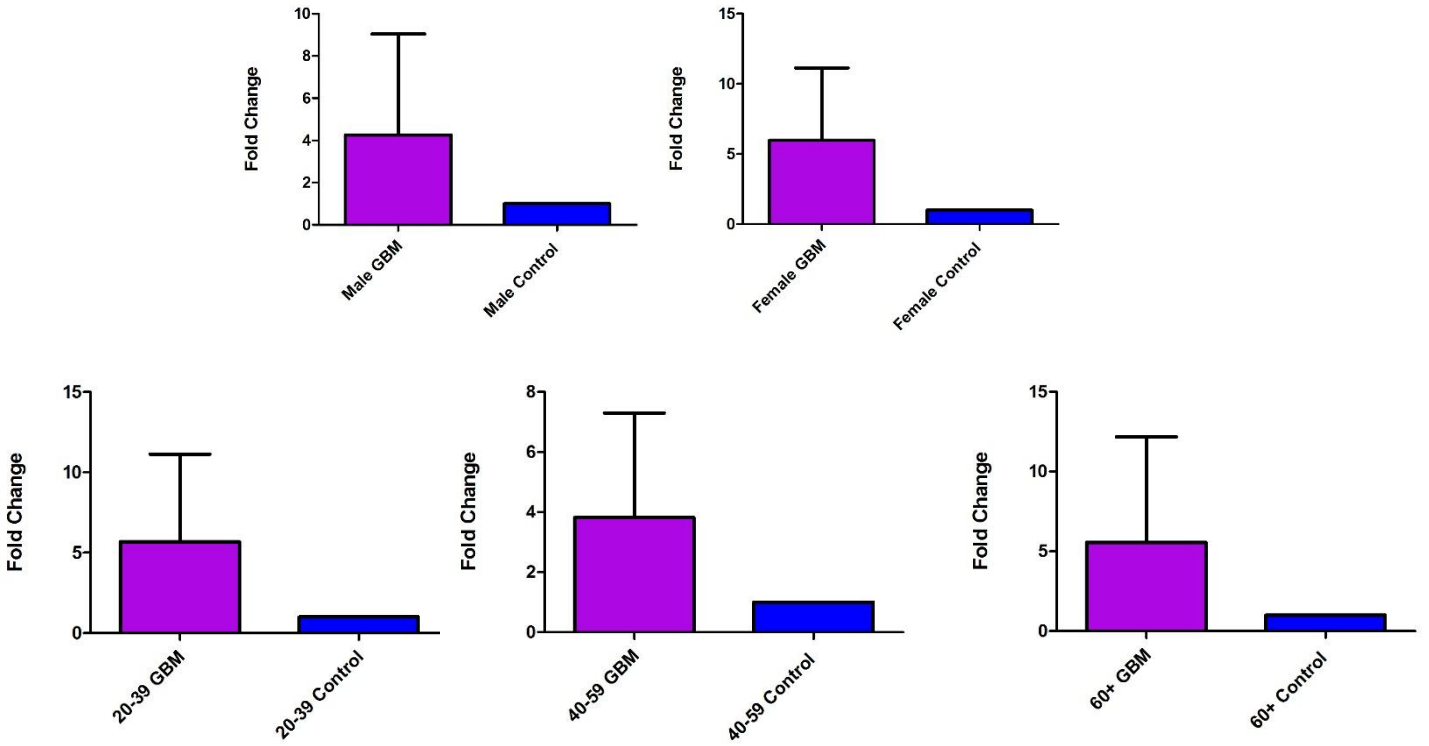
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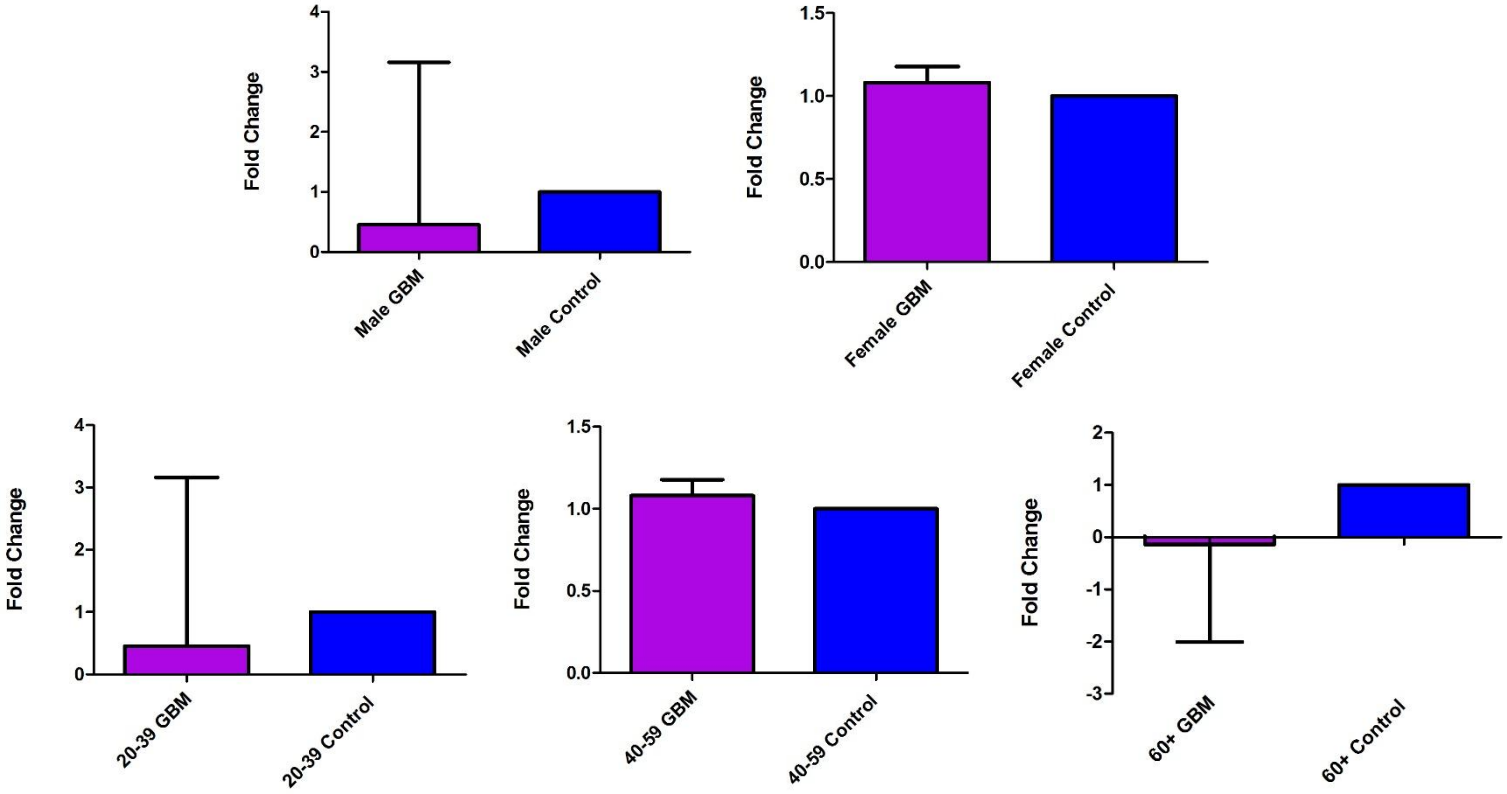
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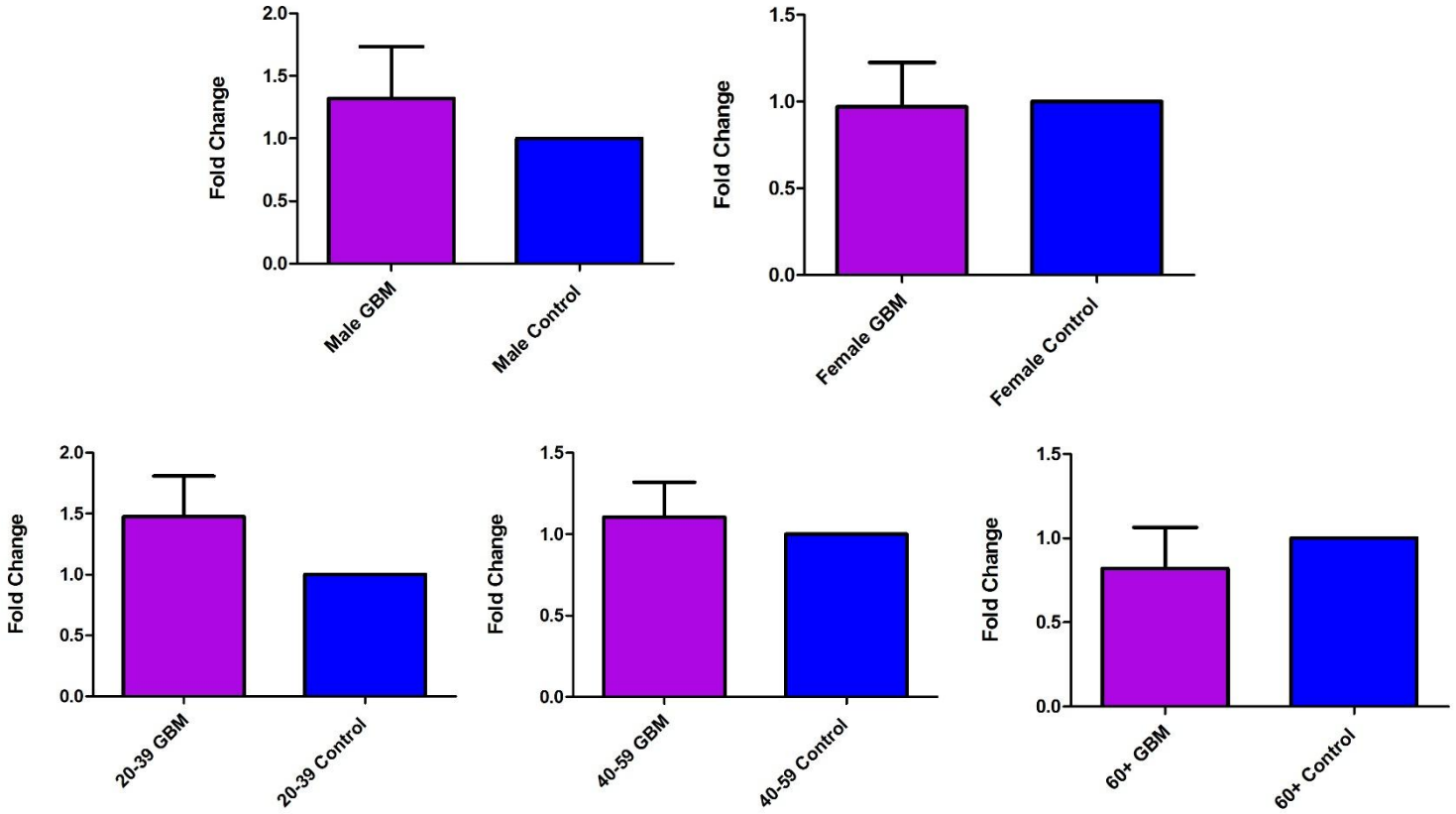
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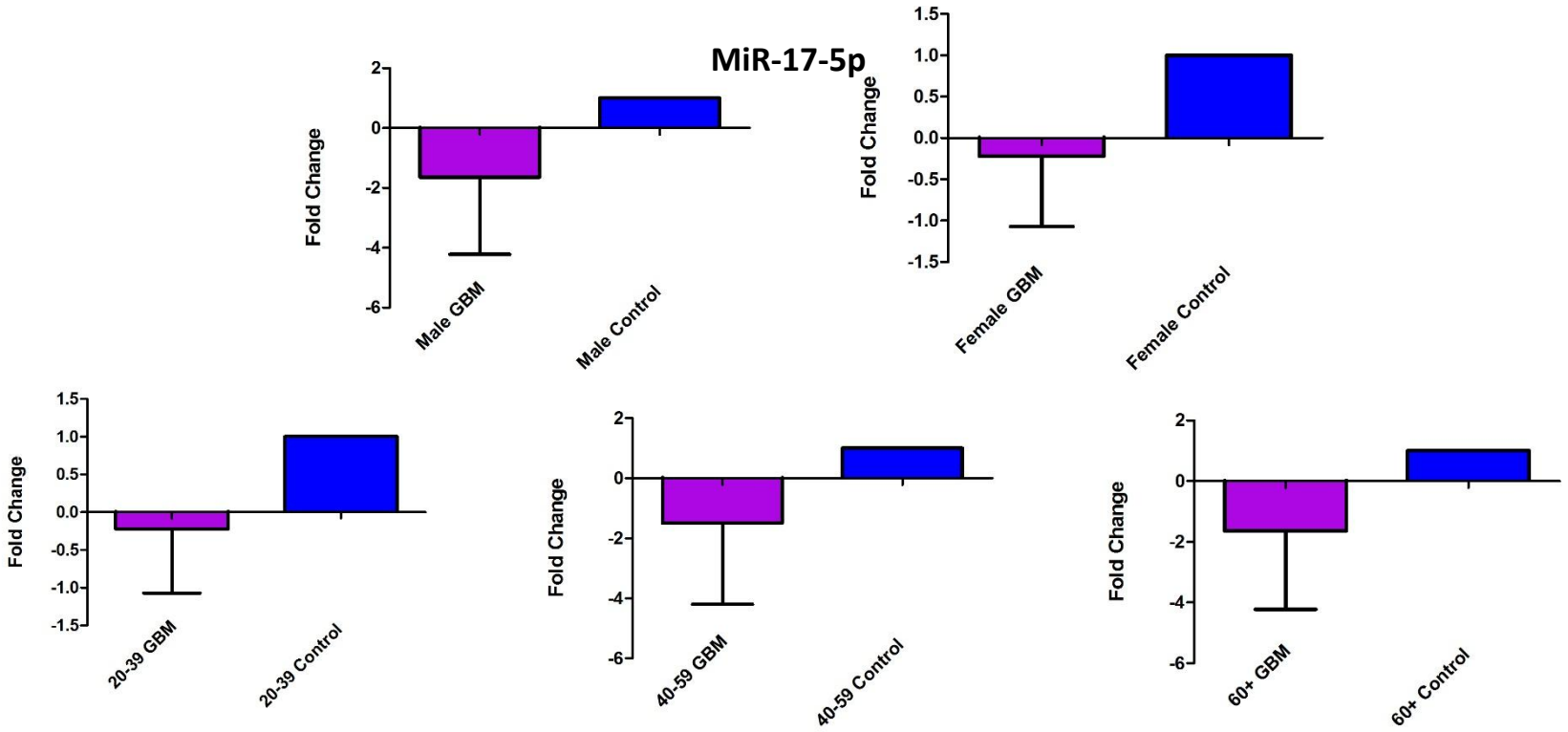
Appendix 4:Reanalysis of non-significant miRNAs



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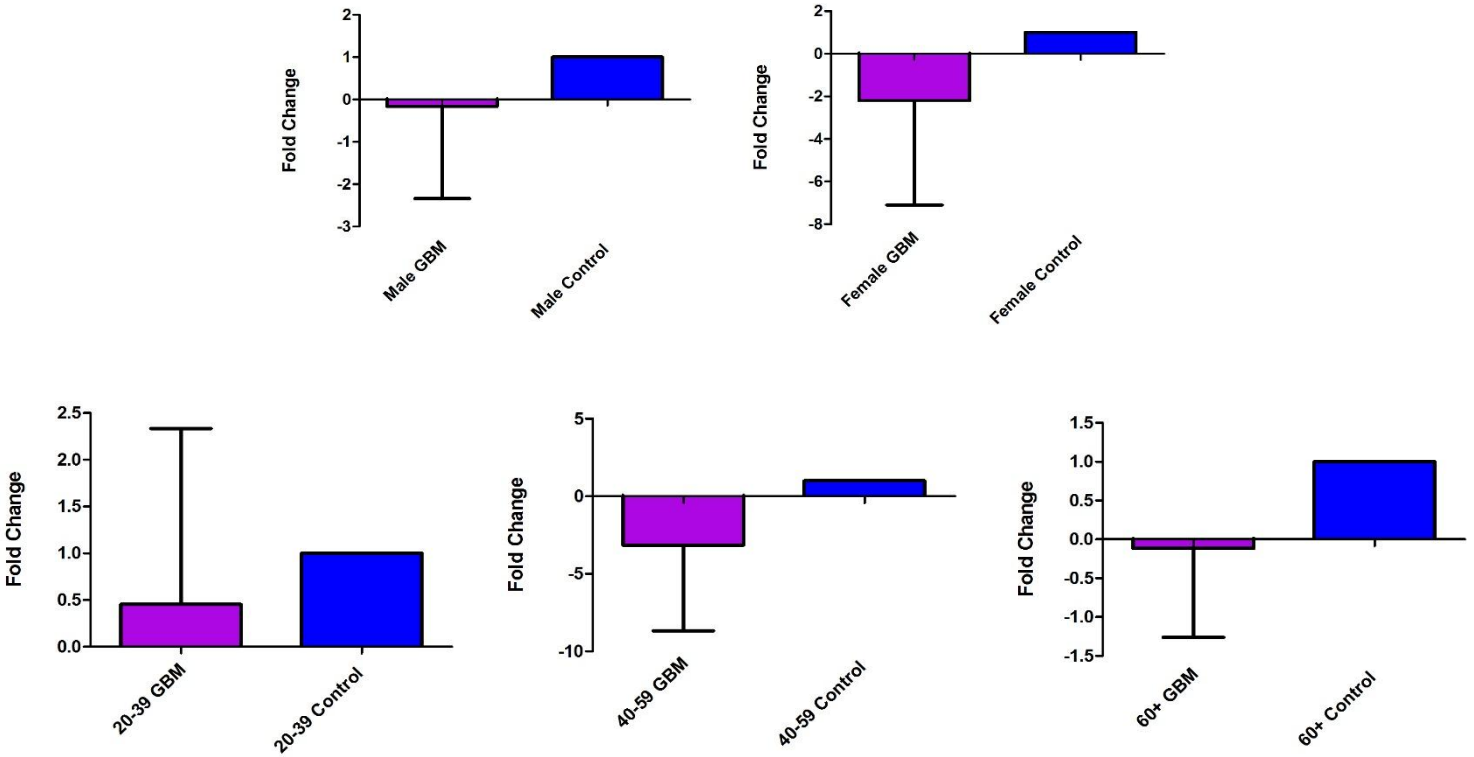
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Fold Change
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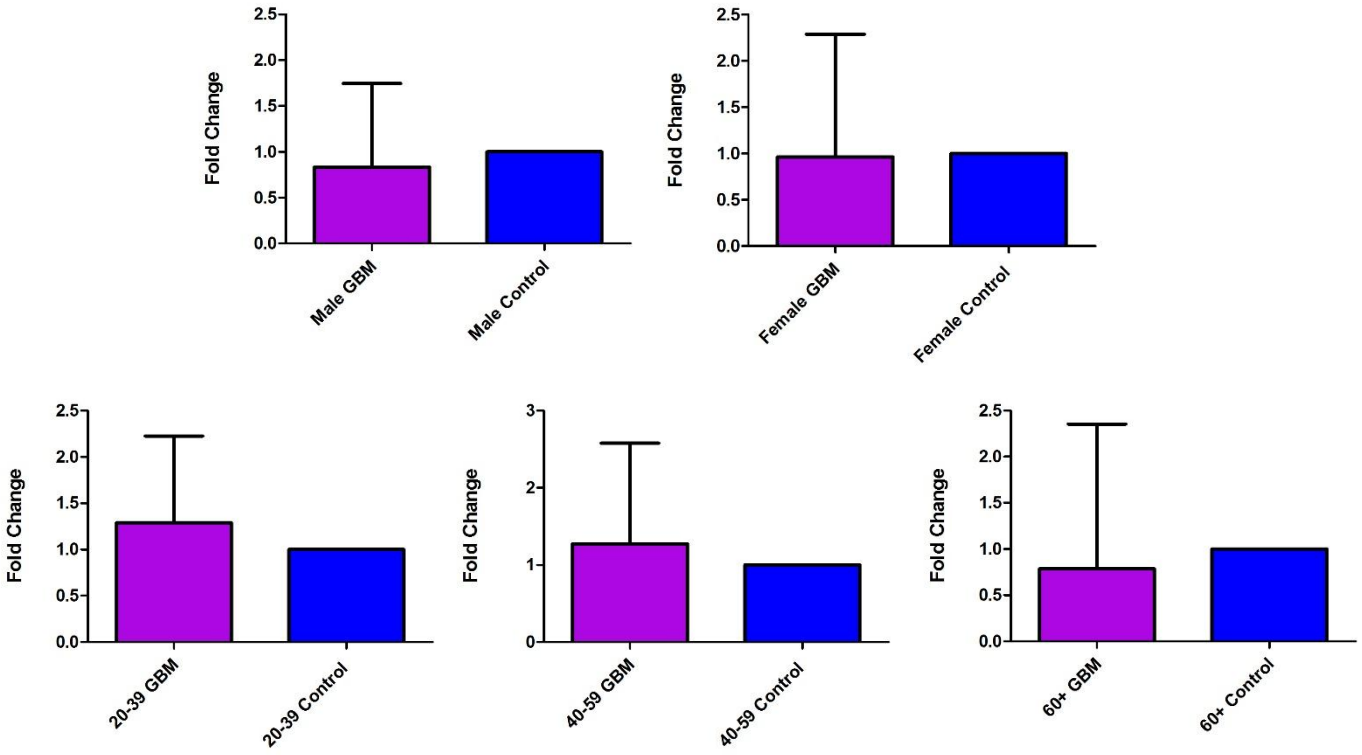
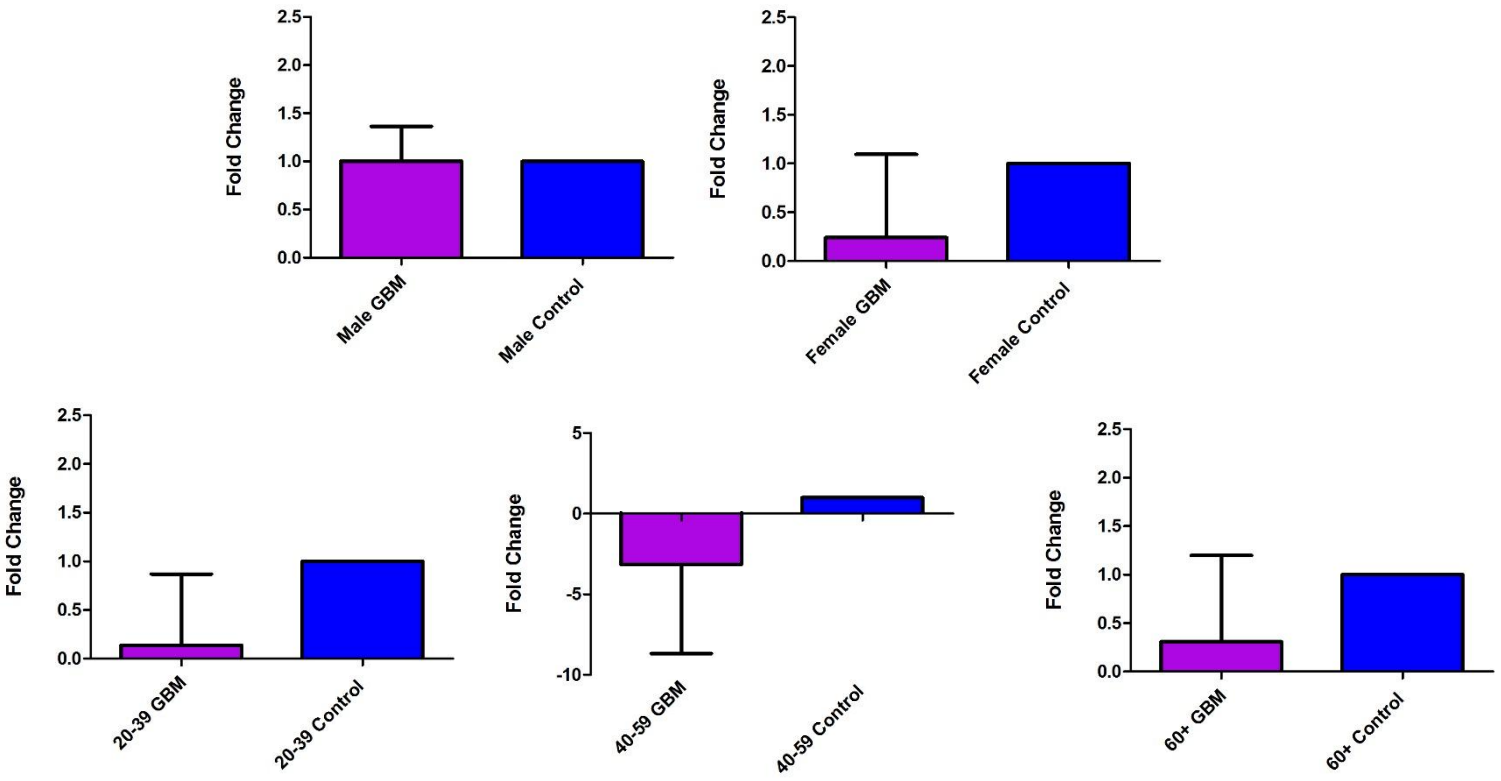
1.0
0.5
0.0
-0.5
-1.0
-1.5
60+ GBM
60+ Control

MiR-29c-3p


Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
Male GBM
Male Control
Female GBM
Female Control
20-39 GBM
20-39 Control
3
2
1
0
40-59 GBM
40-59 Control
60+ GBM
60+ Control

MiR-101-5p


Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
Male GBM
Male Control
Female GBM
Female Control
20-39 GBM
20-39 Control
5
0
-5
-10
40-59 GBM
40-59 Control
60+ GBM
60+ Control

Appendix 5: Comparison of CSF and Serum Total RNA Concentration

CSF Sample	Disease	Concentration (ng/µl)
1223	GBM	13.9
1195	GBM	18.4
1301	GBM	16.0
1300	GBM	58.8
1208	Dermoid Cyst	17.0
1289	NPH	24.3
1293	Dermoid Cyst	68.6
1210	Hydrocephalus	19.7
1234	NPH	44.9
1238	NPH	29.5

Serum Sample	Disease	Concentration (ng/µl)
413	GBM	197.2
927	GBM	1548.1
907	GBM	1034.8
611	GBM	254.9
373	GBM	667.8
1395	Control	1031.8
575	Control	1109.7
594	Control	303.9
591	Control	342.0
1527	Control	865.6

Appendix 6: Example QPCR Data

		AVG Delta(Ct)		Standard Deviation	
		Control Group	Group 1	Control Group	Group 1
A01	hsa-let-7b-5p	-1.480625	-2.088233	0.590399	1.617479
A02	hsa-miR-101-3p	2.905075	1.6818	1.644836	0.63808
A03	hsa-miR-106b-5p	1.550925	1.471767	0.362923	0.741415
A04	hsa-miR-107	4.668825	3.3974	1.267312	1.34556
A05	hsa-miR-10b-5p	4.120325	2.897633	0.715486	1.66676
A06	hsa-miR-124-3p	2.984525	2.1625	1.612239	1.523499
A07	hsa-miR-125a-5p	0.291125	-0.3831	0.394247	0.538157
A08	hsa-miR-125b-5p	-1.901625	-2.219967	0.802248	0.406676
A09	hsa-miR-127-5p	4.668825	3.3974	1.267312	1.34556
A10	hsa-miR-128-3p	2.564625	1.649867	1.222482	1.275635
A11	hsa-miR-129-5p	4.668825	3.3974	1.267312	1.34556
A12	hsa-miR-130a-3p	2.114975	2.737067	0.299071	0.743141
B01	hsa-miR-130b-3p	3.695825	2.905733	0.547195	2.102322
B02	hsa-miR-132-3p	4.346725	3.077233	1.72283	0.876806
B03	hsa-miR-133a-3p	4.668825	3.3974	1.267312	1.34556
B04	hsa-miR-133b	4.668825	3.3974	1.267312	1.34556
B05	hsa-miR-137	4.668825	3.3974	1.267312	1.34556
B06	hsa-miR-138-5p	4.668825	2.759333	1.267312	0.948673
B07	hsa-miR-141-3p	4.314625	3.3974	1.768227	1.34556
B08	hsa-miR-144-3p	1.849125	2.183267	0.247098	0.510937
B09	hsa-miR-146a-5p	4.110575	2.726767	0.477827	0.547367
B10	hsa-miR-146b-5p	3.270975	2.7242	2.140943	0.741592
B11	hsa-miR-148a-3p	3.045075	2.047667	1.029017	1.573276
B12	hsa-miR-149-5p	4.397825	3.3974	1.650564	1.34556
C01	hsa-miR-150-5p	3.491775	3.3974	0.805076	1.34556

C02	hsa-miR-153-3p	4.668825	3.3974	1.267312	1.34556
C03	hsa-miR-15a-5p	4.668825	2.2387	1.267312	0.203821
C04	hsa-miR-15b-5p	4.206375	1.9101	1.433129	0.829889
C05	hsa-miR-16-5p	-0.146875	-0.8589	0.320284	0.346535
C06	hsa-miR-17-5p	2.380775	2.101833	0.675747	0.019412
C07	hsa-miR-17-3p	4.668825	3.3974	1.267312	1.34556
C08	hsa-miR-181a-5p	3.841725	1.279333	0.097616	2.053895
C09	hsa-miR-181b-5p	2.256025	0.359	0.844887	1.05938
C10	hsa-miR-182-5p	4.668825	3.3974	1.267312	1.34556
C11	hsa-miR-183-5p	4.668825	3.3974	1.267312	1.34556
C12	hsa-miR-184	4.668825	3.3974	1.267312	1.34556
D01	hsa-miR-185-5p	4.668825	3.3153	1.267312	1.218508
D02	hsa-miR-187-3p	4.668825	3.3974	1.267312	1.34556
D03	hsa-miR-18a-5p	4.668825	3.284033	1.267312	1.171039
D04	hsa-miR-190a-5p	4.668825	3.300033	1.267312	1.195259
D05	hsa-miR-191-5p	3.451725	1.734733	1.415239	1.201328
D06	hsa-miR-19a-3p	3.210775	2.417433	3.329306	2.209216
D07	hsa-miR-19b-3p	3.435525	2.5718	3.011462	1.429178
D08	hsa-miR-200a-3p	4.668825	3.3974	1.267312	1.34556
D09	hsa-miR-203a-3p	4.668825	3.3974	1.267312	1.34556
D10	hsa-miR-20a-5p	1.056925	0.738933	0.384843	0.70272
D11	hsa-miR-21-5p	-4.043175	-4.647733	0.073362	0.41495
D12	hsa-miR-210-3p	4.668825	3.116033	1.267312	1.088886
E01	hsa-miR-216a-5p	4.668825	3.3974	1.267312	1.34556
E02	hsa-miR-217	4.668825	3.3974	1.267312	1.34556
E03	hsa-miR-218-5p	4.668825	3.3974	1.267312	1.34556
E04	hsa-miR-221-3p	0.363775	-0.834467	0.622219	0.735687
E05	hsa-miR-222-3p	4.426125	2.5094	1.610542	2.125015
E06	hsa-miR-222-5p	4.668825	3.3974	1.267312	1.34556
E07	hsa-miR-23a-3p	0.066775	-1.370467	0.9308	0.689693
E08	hsa-miR-23b-3p	1.695125	0.4042	1.203248	0.934103

E09	hsa-miR-24-3p	-1.931725	-0.702233	1.343043	0.856061
E10	hsa-miR-25-3p	2.189375	0.9708	1.525053	1.488279
E11	hsa-miR-26a-5p	-2.527275	-4.055067	1.259675	1.472554
E12	hsa-miR-27a-3p	0.404025	-0.87	0.5298	0.899326
F01	hsa-miR-296-5p	4.668825	3.301467	1.267312	1.369241
F02	hsa-miR-29a-3p	0.336375	-0.657867	0.207854	0.78976
F03	hsa-miR-29b-3p	3.177925	0.857533	0.986732	1.746567
F04	hsa-miR-29c-3p	0.083475	-1.229533	0.511769	0.812761
F05	hsa-miR-30b-5p	2.338725	0.958	0.658776	1.168934
F06	hsa-miR-30c-5p	1.653375	0.1685	1.312921	1.141486
F07	hsa-miR-31-5p	4.272925	2.557567	0.707425	1.34549
F08	hsa-miR-320a	1.691325	0.1932	1.083323	1.11365
F09	hsa-miR-323a-5p	4.631125	3.172367	1.213996	1.007027
F10	hsa-miR-324-5p	4.401375	2.9899	0.889081	1.346387
F11	hsa-miR-326	4.668825	3.3974	1.267312	1.34556
F12	hsa-miR-328-3p	0.525925	-2.1129	2.135922	2.011041
G01	hsa-miR-331-5p	4.668825	3.3974	1.267312	1.34556
G02	hsa-miR-335-5p	4.668825	3.171833	1.267312	1.006269
G03	hsa-miR-34a-5p	4.650075	2.415867	1.293829	1.312732
G04	hsa-miR-425-5p	4.293275	2.688	0.736204	1.895129
G05	hsa-miR-451a	-1.348675	-1.716733	0.480019	1.65848
G06	hsa-miR-486-5p	2.868275	1.164033	0.997834	1.434588
G07	hsa-miR-7-5p	4.516975	2.764467	1.052564	0.587371
G08	hsa-miR-9-5p	-1.104375	-3.2088	1.255857	1.81362
G09	hsa-miR-9-3p	1.304375	-0.432267	0.608784	1.489972
G10	hsa-miR-92a-3p	0.492525	-1.591867	0.246533	0.912989
G11	hsa-miR-93-5p	3.606575	2.3882	0.234936	1.104773
G12	hsa-miR-96-5p	4.668825	3.100533	1.267312	1.479333