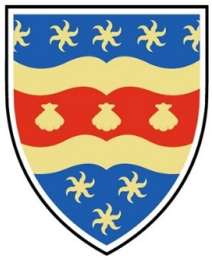
2020

ROLE OF miRNome IN MENINGIOMA TUMOURS: IDENTIFICATION OF POTENTIAL MOLECULAR BIOMARKERS AND THERAPEUTIC TARGETS FOR HIGH-GRADE MENINGIOMA

Negroni, Caterina



UNIVERSITY OF PLYMOUTH

ROLE OF miRNome IN MENINGIOMA TUMOURS: IDENTIFICATION OF POTENTIAL MOLECULAR BIOMARKERS AND THERAPEUTIC TARGETS FOR HIGH-GRADE MENINGIOMA

By

CATERINA NEGRONI

A thesis submitted to the University of Plymouth

in partial fulfilment of the degree of

Doctor of Philosophy

School of Medicine and Dentistry

December 2019

Acknowledgements

First of all, I would like to thank my Director of Studies, Prof Oliver Hanemann, for giving me the opportunity to undertake my PhD in his group and for all his time and constructive comments throughout this journey.

My most sincere thanks go to my supervisor, Dr Daniele Baiz, for your guidance and support during the whole of my PhD; I really appreciated your kindness and patience in training me to be a research scientist and in presenting me challenges that helped me grow.

A huge acknowledgement to all the Hanemann group, past and present: Sara, Claire, Emanuela, Sylwia, Boh, Lucy, Foram, Bora, Jemma, Yeasmin, and Laurien; along with Laura and Liyam you became my second family, giving me support, advice and making my time in lab more pleasant.

Outside of the lab, I am extremely grateful to my family for always being there for me, believing in me and giving me the strength to keep going. Firstly, thanks to Mum and Dad; none of this would have been possible without you. Moving to Plymouth for my PhD was not an easy decision to make, but you have always been by my side and helped me through this huge change in my life and I cannot thank you enough for this. To my partner in crime, my sister Cristina, who I can always count on and never stopped believing in me.

A special thank you to Nonno, you have always been my number one fan and given me so much strength. Sadly we had to part ways, but I know you will always be by my side, guiding my way.

I think it is difficult to find real friends but I am lucky enough to have some back home. Thanks to Andrea, Alessandro, Davide, Sara, Viviana, and Valentina; we do not get to see each other very often, but every time we meet it is like we never parted.

Last but not least, thanks to my partner, Rob. I think words cannot describe how grateful I am for having you in my life; I went through some really hard times during this journey, especially in my personal life, and you have always been there to put a smile on my face and keep me going.

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

This study was financed with the aid of a studentship from the University of Plymouth.

Presentations at conferences:

Brain Tumor Meeting. Berlin, Germany. May 23-24, 2019. Poster presentation.

Making it Personal: Cancer Precision Medicine. Bergamo, Italy. November 5-7, 2018. Poster presentation.

EACR 25th Biennial Congress. Amsterdam, Netherlands. June 30 – July 3, 2017. Poster presentation.

Word count of main body of thesis: 49634

Signed

Date

Caterina Negroni

ROLE OF miRNome IN MENINGIOMA TUMOURS: IDENTIFICATION OF POTENTIAL MOLECULAR BIOMARKERS AND THERAPEUTIC TARGETS FOR HIGH-GRADE MENINGIOMA

Abstract

Meningioma is the most common intracranial tumour, accounting for one third of all primary brain and central nervous system (CNS) tumours. According to the World Health Organization (WHO), meningiomas are classified as grade I, II, and III based on the histological features of tumours, with higher grades associated with greater morbidity and mortality. In most cases, meningioma can be managed by surgery alone; however, adjuvant treatment may be required in case of recurrent or higher-grade tumours. To date, chemotherapy has proven ineffective in treating meningioma patients, highlighting the need to identify novel molecular biomarkers and therapeutic targets, establishing effective non-invasive treatments. The aim of this project is to elucidate microRNA (miRNA)-associated post-translational mechanisms involved in the regulation of Cyclin D1, which has been suggested as a potential prognostic biomarker for meningioma. By focusing on the analysis of specific miRNAs by comparing WHO I, II, and III meningioma tissues and matched blood samples, this study identified potential diagnostic biomarkers circulating in peripheral blood to support the discovery of early tumour recurrence. Moreover, determination of the factors responsible for driving deregulation of miRNA candidates may generate potential therapeutic targets.

Overexpression of Cyclin D1 was found to correlate with downregulation of the miR-497~195 cluster *via* the GATA binding protein 4 (GATA-4), a zinc finger transcription factor upregulated in malignant meningioma. Studies in our cell culture model of meningioma demonstrated that high levels of GATA-4 in malignant meningioma cells augment cell viability by controlling the expression of the miR-497~195 cluster, increasing the expression of Cyclin D1, D2, D3, and E1. Consistently, the small-molecule inhibitor NSC140905 reduced GATA-4 activity in malignant meningioma KT21-MG1 cells, restoring miR-497~195 levels. This resulted in decreased cell viability, and reduction of Cyclin D1, D2, D3, and E1 expression. Furthermore, analysis of miR-497~195 in circulating exosomes, derived from higher-grade (grade II and III) meningioma patients, showed lower levels of the miR-497 compared to those of benign origin (grade I).

In conclusion, these findings suggest that GATA-4 is a novel potential therapeutic target for malignant meningioma correlating with the miR-497~195 cluster. Moreover, analysis of circulating exosome cargo suggests miR-497 to be a potential non-invasive biomarker for higher-grade meningioma tumours.

Table of Contents

Table of Figures

Table of Tables

List of abbreviations

ADCC	Antibody-dependent cellular toxicity
AGO	Argonaute
AKT1/3	AKT serine/threonine kinase 1/3
ANOVA	Analysis of variance
ATRA	All-*trans*-retinoic acid
AUC	Area under the curve
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
CLL	Chronic lymphocytic leukaemia
BSA	Bovine serum albumin
CCND1-2-3, E1	Cyclin D1-2-3, E1
CD9	Cluster of differentiation 9
CD63	Cluster of differentiation 63
CDK2/4/6	Cyclin-dependent kinase 2/4/6
CDKN2A/B	Cyclin dependent kinase inhibitor 2A/B
CI	Combination index
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CT	Computerised tomography

DAB	3,3'-Diaminobenzidine
DAL-1	Differentially expressed in adenocarcinoma of the lung
DGCR8	DiGeorge syndrome critical region 8
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTATATE	DOTA-(Tyr^3)-octreotate
DPI	Dots per inch
ECL	Enhanced chemical luminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EORTC	European organization for research and treatment of cancer
ER	Endoplasmic reticulum
ETS	E-twenty-six
EV	Extracellular vesicles
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FDA	Food and drug administration
FOG2	Friend of GATA 2

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-4/5/6	GATA-Binding protein 4/5/6
GFP	Green fluorescent protein
GFM	Growth factor medium
GM130	Golgi matrix protein
GTR	Gross total resection
Gy	Gray
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylases
HH	Hedgehog
HMC	Human meningeal cells
HPF	High power field
HPV	Human papilloma virus
H-RAS	Harvey rat sarcoma viral oncoprotein
H-RAS^{V12}	H-RAS variant with Valine at position 12 instead of Glycine
HRP	Horseradish peroxidase
HU	Hydroxiurea
IHC	Immunohistochemistry
INF	Interferon
KLF4	Krüppel-like factor 4
LA	Laminin

LOH	Loss of heterozigosity
MEKK3	Mitogen-activated protein/ERK kinase kinase 3
MEN1	Multiple endocrine neoplasia type 1
Merlin	Moesin Ezrin Redixin like protein
miRNA	microRNA
MN	Meningioma
MOI	Multiplicity of infection
MOT	Molecular target identification
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian target of rapamycin complex 1/2
MVB	Multivesicular body
NF	Neurofibromatosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NMT	Normal meningeal tissue
NSCLC	Non-small cell lung cancer
Pasha	Partner of Drosha
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase-chain reaction
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death ligand 1
PDMN	Patient-derived meningioma cells
Pen/Strep	Penicillin/streptomycin
PET	Positron emission tomography
PFS	Progression free survival
PFS6	Progression free survival at 6 months
PI3K	Phosphatidylinositol-3-kinase
PI3KCA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PLL	Polylysine
POLR2A	RNA polymerase II subunit A
PPIi	Protein-protein interaction inhibitors
Pre-miRNA	precursor miRNA
Pri-miRNA	primary miRNA
PTCH1	Protein patched homolog 1
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
Rb	Retinoblastoma protein
pRB	Phosphorylated retinoblastoma protein

RIPA	Radioimmunoprecipitation buffer
RISC	RNA-induced silencing complex
RLC	RISC loading complex
ROC	Receiver operating characteristic
RPM	Revolutions per minute
RT	Radiation therapy
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1
SMARCE1	SWI/SNF related matrix-associated actin-dependent regulator of chromatin subfamily E member 1
SMO	Signalling member smoothened
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
snRNA	Small nuclear RNA
SRC	Proto-oncogene Tyrosine-protein kinase SRC
SRS	Stereotactic radiosurgery

SSTR	Somatostatin receptor type IIA
STR	Subtotal resection
SUFU	Suppressor of fused homolog
SWI/SNF	SWItch/Sucrose Non-Fermentable
TBS	Tris-buffered saline
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
TRAF7	TNF receptor-associated factor 7
UTR	Untranslated region
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

1 Introduction

1.1 Background of the study

Meningiomas represent the most common intracranial tumour, accounting for one third of all primary brain and central nervous system (CNS) tumours (Wiemels, Wrensch & Claus, 2010). Despite their genetic mutations being well characterised, therapeutic approaches have very limited options, due to lack of specific biomarkers.

In most cases, meningiomas can be managed by surgery alone or with adjuvant radiotherapy. However, a number of complicating factors, such as tumour location and aggressive histological features, lead to disease progression or tumour recurrence with associated patient morbidity and mortality (Marosi *et al.*, 2008; Norden *et al.*, 2010; Chamberlain, 2012; Kaley *et al.*, 2015; Rogers *et al.*, 2015; Rogers *et al*., 2016; Kaley *et al.*, 2015).

In addition, current meningioma grading is solely based on histopathological observation of tumour biopsies, a method subjected to inter-observer variability and lacking of precise definitions, which makes the practical application difficult (Willis *et al.*, 2005; Louis *et al.*, 2016).

Nevertheless, there have been some attempts to find molecular signatures that could aid meningioma classification. For example, it has been shown that Cyclin D1 (CCND1) is overexpressed in meningioma tumours compared with healthy controls. Its expression level correlates with the risk of recurrence, and Cyclin D1 knockdown led to a decrease in cell proliferation, promoted apoptosis and

attenuated the invasive capacity in two different meningioma cell lines (Alama *et al.*, 2007; Milenkovic *et al.*, 2008; Cheng *et al.*, 2015).

Moreover, a number of studies recently focused their attention on microRNAs (miRNAs), but we still need to identify reliable molecular biomarkers to ameliorate tumour diagnosis and prognosis (Zhi *et al.*, 2013; Ludwig *et al.*, 2015; Wang *et al.*, 2015; Zhi *et al.*, 2016; El-Gewely *et al.*, 2016; Ludwig *et al.*, 2015).

This study used a biased approach to the identification of miRNA signatures that could be involved in meningioma progression; by literature review, I identified proteins that have been suggested as potential biomarkers for meningioma, and the miRNAs that are involved in their regulation. I thus studied meningioma specimens encompassing all grades in order to understand if the expression levels of the miRNA candidates correlated with those of the suggested protein biomarkers, and to investigate their regulation. Using an *in vitro* system, I performed functional studies and drug testing to assess whether the identified candidates could be suitable therapeutic targets. Furthermore, I studied the expression levels of the miRNA candidates in exosomes isolated from meningioma patients' serum samples in order to understand whether they are eligible as circulating biomarkers.

In the following introduction, the definition of biomarkers and their use in neuro-oncology will be discussed, along with current meningioma literature and recent advances in studies for the identification of circulating biomarkers.

1.2 Biomarkers in neuro-oncology

Biomarkers play a crucial role in neuro-oncology, both in routine clinical care, forming the basis for a precision medicine approach, and in therapeutic development, to improve and speed up the approval of new therapies (Cagney *et al.*, 2018). However, the term “biomarker” and its subgroupings are often used improperly and this can slow down their adoption, proper application, and significance.

The BEST (Biomarkers, EndpointS, and other Tools) glossary was thus developed in 2016 by the FDA and the National Institutes of Health (NIH) to standardize the nomenclature and provide clarity in the biomarker field (Cagney *et al.*, 2018). According to these guidelines, a biomarker is described as “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers but a biomarker is **not** an assessment of how an individual feels, functions, or survives.” [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. Biomarkers can be measured anywhere along the clinical continuum, from pre-diagnosis of disease, pre-treatment, to the post-treatment phase (Fig. 1.1) (Cagney *et al.*, 2018).

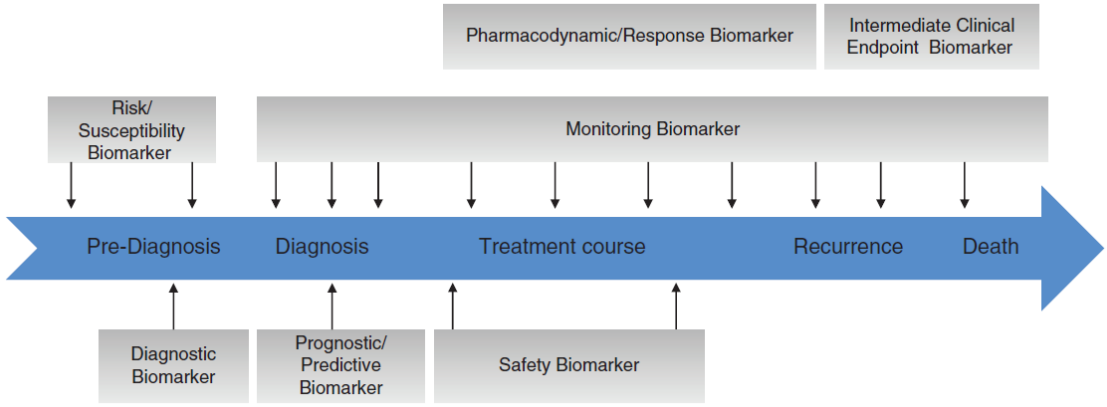


Figure 1.1 Biomarkers along the clinical continuum. Depending on their function, biomarkers can be classified in seven types: susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamics/response, and safety biomarkers. Figure from (Cagney *et al.*, 2018).

According to the BEST guidelines, we can distinguish seven types of biomarkers: susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic/response/endpoint, and safety biomarkers.

A susceptibility/risk biomarker is defined as a characteristic that indicates the potential for developing a disease or a medical condition in an individual who does not currently have a clinically apparent disease or medical condition [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. Examples of susceptibility biomarkers in neuro-oncology are inherited genetic disorders (Ohgaki & Kleihues, 2005; Schwartzbaum *et al.*, 2006), history of ionizing radiation (biomarkers that correlate with the absorbed dose of ionizing radiations or "biodosimeters") (Rana *et al.*, 2010), DNA repair gene polymorphisms (Adel Fahmideh *et al.*, 2014), single-nucleotide polymorphism

(Jenkins *et al.*, 2012; Melin *et al.*, 2017), and atopy (Linos *et al.*, 2007; Calboli *et al.*, 2011).

A diagnostic biomarker is described as a feature used to detect or confirm the presence of a disease or condition of interest, or to identify individuals with a subtype of the disease [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. This type of biomarker is frequently used in neuropathology and is increasingly vital for the classification of brain tumours; in fact, the 2016 World Health Organization classification (Louis *et al.*, 2016) included several molecular parameters in addition to histopathology. The advantage of using diagnostic biomarkers is the possibility to define different patient populations, thus increasing the chances that these markers could function as predictive or prognostic markers to support precision medicine, and make therapeutic signals easier to detect (Cagney *et al.*, 2018).

A monitoring biomarker is defined as a parameter serially measured to assess the status of a disease or medical condition (*e.g.* imaging). It is used to assess safety and toxicity (*e.g.* platelet and blood count), or for evidence of exposure to, or effect of, a medical product or an environmental agent (*e.g.* drug serum levels) [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. Blood is the most easily accessible tissue source for monitoring biomarkers, and it is frequently used to assess the disease status of many malignancies; however, in the neuro-oncology context, blood-based monitoring biomarkers might be more problematic due to the presence of the blood-brain barrier (BBB), which blocks reliable shedding of tumour-related biomarkers into circulating blood (Cagney *et al*., 2018). Interestingly, meningiomas are outside the BBB, meaning this limitation is less of an issue in this tumour type.

Nonetheless, the potential large benefits outweigh the drawbacks, thus supporting areas of research into blood-based biomarkers in neuro-oncology, including circulating tumour cells, exosomes (Shao *et al.*, 2012), and microRNAs (Qiu *et al.*, 2013; Akers *et al.*, 2013). Besides disease monitoring, these biomarkers can also be useful as response biomarkers or as diagnostic, prognostic and predictive biomarkers (Cagney *et al.*, 2018).

Prognostic biomarkers are used to identify the likelihood of a clinical event, disease recurrence, or progression, based on the natural history of the disease [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. Therefore, this type of biomarkers is generally associated with a specific outcome, including progression-free survival (PFS) or overall survival (OS) (Cagney *et al.*, 2018).

Predictive biomarkers are used to discover people who are more likely to experience a favourable or adverse effect from exposure to a medical product or an environmental agent [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016].

The aim of pharmacodynamic/response biomarkers is to show that a biological response has occurred in an individual who has been exposed to a medical product or environmental agent [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. To date, blood-based pharmacodynamic biomarkers are the most common example in neuro-oncology, and include measurement of peripheral blood mononuclear cells by Western blot *e.g.* to determine the inhibition of targets such as pAKT and pS6 following small-molecule AKT inhibitor treatment (Fouladi *et al.*, 2014).

Pharmacodynamic biomarkers are usually related to the biology of the experimental therapy, thus being more therapy-specific than response biomarkers (Cagney *et al.*, 2018). Sometimes, changes in pharmacodynamic biomarkers are thought to be a necessary (if not sufficient) result for an experimental therapy to be effective. In such cases, they can be important early phase clinical trial endpoints as signals of biologic activity (Alexander & Cloughesy, 2017). Changes in a pharmacodynamic biomarker may provide confidence in further testing or suggest candidates for combinations when later-stage endpoints are not met (Cagney *et al.*, 2018).

Safety biomarkers constitute parameters measured before or after an exposure to a medical product or environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect [*BEST (Biomarkers, EndpointS, and other Tools) Resource*. Maryland. 2016]. The most common safety biomarker used in neuro-oncology is weekly complete blood counts in patients receiving chemoradiation to monitor for myelosuppression (Gerber *et al.*, 2007).

1.3 Meningioma: origin and classification

Meningioma originates from the meninges, the covering layers of the brain and the spinal cord (Whittle *et al.*, 2004; Rogers *et al.*, 2015), which are composed of three membranes: the *dura mater*, the thickest layer situated in close proximity to the skull and vertebrae, the arachnoid *mater*, the middle layer which has a web-like morphology, and the *pia mater*, the innermost layer of the meninges which lines the brain and the spinal cord.

In particular, meningioma is thought to arise either from the arachnoidal cap cells, a subset of arachnoid cells located at the apex of the arachnoid *villi* of the arachnoid *mater*, which are involved in the resorption of the cerebrospinal fluid (CSF) (Marosi *et al.*, 2008), or from dural border cells (Boetto *et al.*, 2018) (Fig. 1.2).

Most meningioma tumours can be found within the convexity, parasagittal and sphenoid regions. Less frequently, meningiomas can be found at the cerebellopontine angle, optic nerve sheath, and the choroid plexus. Spinal meningiomas are less frequently observed than skull-based tumours and, very rarely, meningioma tumour formation may occur at ectopic sites such as the ear, mandible, foot and lung (<1%) (Hallinan, Hegde & Lim, 2013).

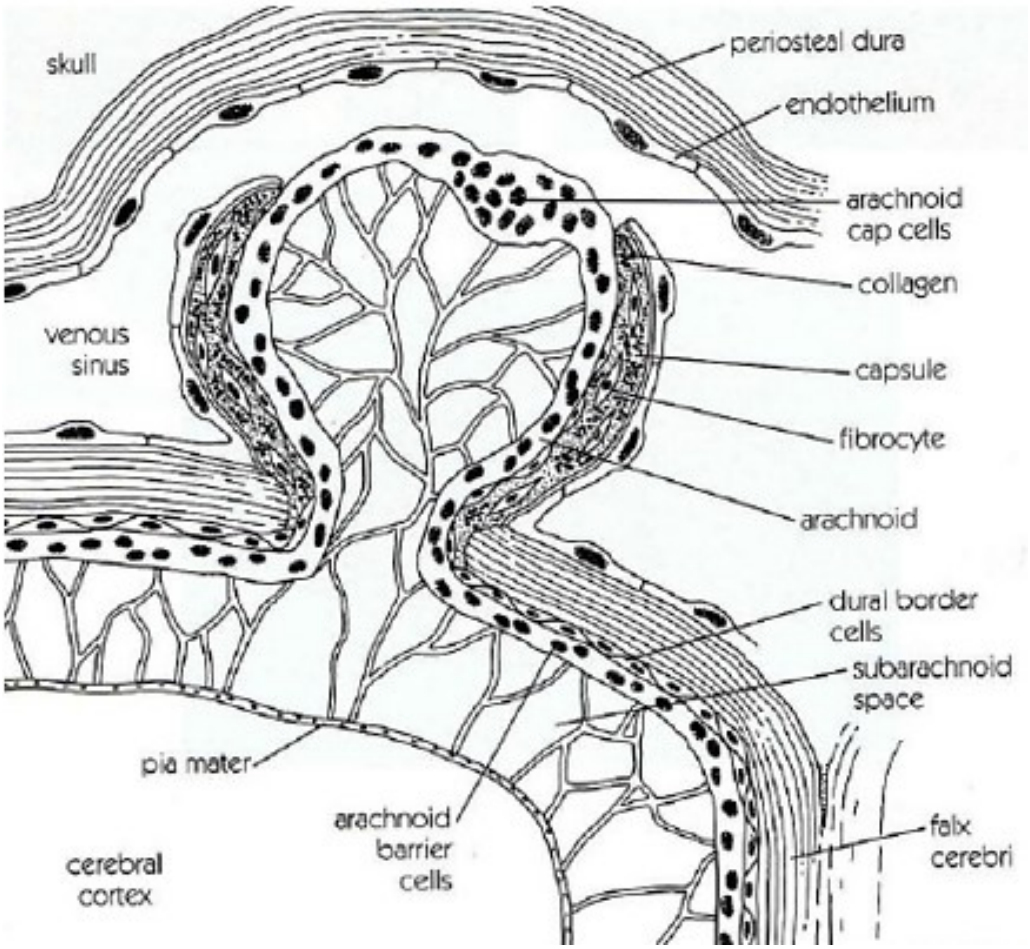


Figure 1.2 Schematic illustration of the meninges. Representation of the three meningeal layers: *dura mater*, arachnoid *mater*, and *pia mater*. Figure from https://operativeneurosurgery.com/doku.php?id=arachnoid_cap_cell.

Meningioma classification is based on histopathological characterization of the tumour following the World Health Organization (WHO) guidelines, which encompass three grades and 15 histological subtypes (Louis *et al.*, 2016).

The vast majority of meningiomas (~80%) are slow-growing WHO grade I tumours, with a generally benign nature and a low mitotic count (Riemenschneider, Perry & Reifenberger, 2006). Grade I meningiomas can be further classified in nine histological subtypes, of which meningothelial, fibrous

and transitional are most commonly diagnosed (Riemenschneider, Perry & Reifenberger, 2006). WHO grade II tumours, also termed atypical meningioma, represent approximately 18-20% of all meningiomas and encompass two histologic variants, clear-cell and chordoid tumours (Riemenschneider, Perry & Reifenberger, 2006; Commins, Atkinson & Burnett, 2007; Louis *et al.*, 2016). Grade II meningiomas are defined by one or more of the following four criteria: chordoid or clear-cell subtype, four to 19 mitoses per ten high power fields (HPFs), brain infiltration, and three or more of the following five histologic features; small cells with a high nuclear-to-cytoplasmic ratio, hypercellularity, prominent nucleoli, sheet-like growth, and *foci* of spontaneous necrosis (Backer-Grondahl, Moen & Torp, 2012). Lastly, WHO grade III meningioma tumours are least common, accounting for just 1-3% of all meningiomas, and are referred to as anaplastic or malignant in histology. They display obvious malignant cytology, resembling that of sarcoma or carcinoma, or a high mitotic index of 20 or more mitoses per 10 HPFs (Louis *et al.*, 2016). Among WHO grade III tumours, two rare subtypes can be identified; rhabdoid and papillary. The former are comprised predominantly of rhabdoid cells, resulting in large tumour cells with irregularly placed nuclei and an abundant eosinophilic cytoplasm, while the latter are characterised by a perivascular pseudopapillary pattern in which tumour cells appear to cling to blood vessels (Louis *et al.*, 2016).

A schematic diagram of meningioma subtypes and their associated WHO grades can be found below (Fig. 1.3).

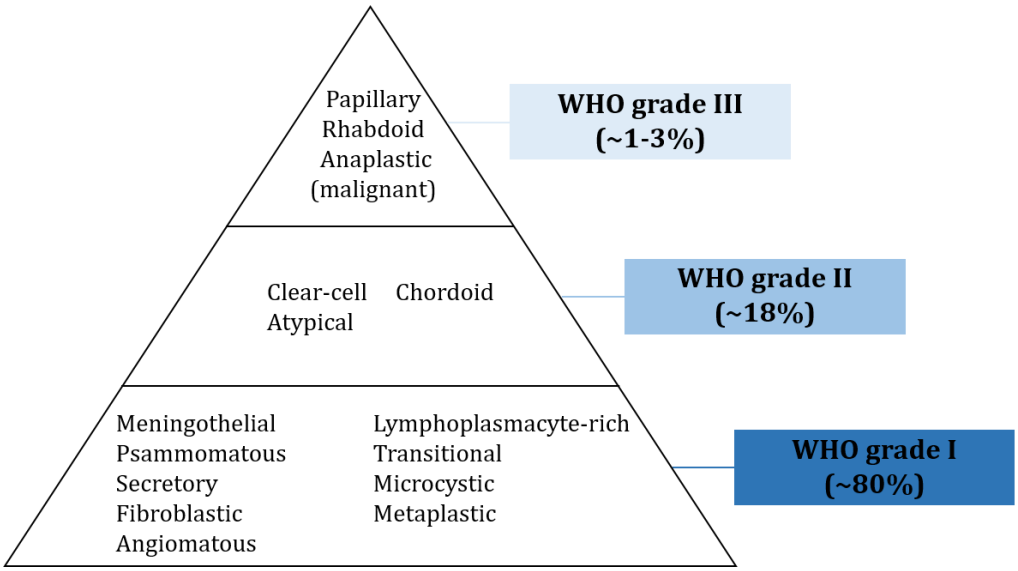


Figure 1.3 Histological variants of meningioma classification. Grade I meningiomas account for 80% of all meningioma tumours, and are slow-growing lesions with a low recurrence risk, while the remaining 20% of meningioma tumours are classified as grade II or III. These high-grade tumours are generally more aggressive in nature and they each consist of three histological subtypes (Commins, Atkinson & Burnett, 2007; Louis *et al.*, 2016).

1.4 Clinical presentation

Many small meningiomas can be asymptomatic, with tumours often diagnosed as a result of imaging for other purposes (Mawrin, Chung & Preusser, 2015). In these cases, little is known about the natural history and growth patterns of the tumours, thus presenting a dilemma for therapeutic intervention (Chamoun, Krisht & Couldwell, 2011).

When a patient experiences meningioma symptoms, they depend on the location of the tumour and its relationship with the surrounding anatomy.

Commonly, patients present with seizures, papilledema, headache, dizziness, nausea, vomiting, drowsiness, or focal neurological deficits (*e.g.* progressive weakness or paralysis of one side of the body) (Whittle *et al.*, 2004; Wu *et al.*, 2018).

1.5 Incidence and risk factors

Meningiomas constitute 37.1% of all brain tumours, and they are more common in women than men, with a female-to-male ratio of 2:1 and 9:1 for intracranial and spinal meningiomas in adults, respectively (Marosi *et al.*, 2008; Ostrom *et al.*, 2018). The annual occurrence for meningioma is estimated to 2-7/100,000/year for women and 1-5/100,000/year for men (Marosi *et al.*, 2008).

Meningiomas show a dramatic increase in incidence in the sixth and seventh decade of life, with an average age at diagnosis of 66, whereas they are one of the least common tumours in children aged 0-14 years (Ostrom *et al.*, 2018), representing 0.4-4.1% of pediatric tumours (Tufan *et al.*, 2005) and, in contrast to adult meningioma, have a reported male predominance (Ostrom *et al.*, 2018).

In adolescents and young adults (15-39 years of age), meningiomas account for approximately 16% of all primary brain and CNS tumours (Ostrom *et al.*, 2015).

Deletions or germline mutations of the tumour suppressor gene *Neurofibromatosis 2* (*NF2*; long arm of chromosome 22, 22q12) represent established risk factors for meningioma development. The associations between monosomy 22 and meningiomas was first studied in patients with NF2, which is a dominantly inherited genetic disorder; NF2 patients commonly

present with bilateral vestibular schwannoma, a hallmark of NF2, alongside multiple meningiomas, and other nervous system tumours (Choy *et al.*, 2011).

Meningioma can also arise in patients affected by other hereditary syndromes, such as Cowden, Gorlin, Li-Fraumeni, Turcot, Gardener, von Hippel-Lindau, and multiple endocrine neoplasia type I (MEN1) (Louis & von Deimling, 1995; Asgharian *et al.*, 2004). However, given the rarity of meningioma occurrence in these conditions, it is still not clear whether these disorders are causal or merely coincidental (Riemenschneider, Perry & Reifenberger, 2006).

Another well established risk factor for meningioma is exposure to ionizing radiation. In fact, as little as 8 Gray (Gy) was found responsible for single or multiple meningiomas with a life time risk increase of 2.3% after a latency period of 35 years (Marosi *et al.*, 2008; also reviewed in Braganza *et al.*, 2012).

The higher incidence of meningiomas in women, the worsening of meningioma symptoms especially during periods of relative progesterone excess such as the luteal phase of the menstrual cycle and pregnancy, and their association with breast and genital cancers, strongly suggests oestrogen and progesterone dependency of these tumours (Collins, 2004). However, there is no definitive evidence of hormonal influence on tumour growth, as the proportion of meningiomas expressing progesterone receptors is not significantly different between males and females (Korhonen *et al.*, 2006), and the effects of pregnancy on meningioma growth are thought to be due to temporary hemodynamic changes (Lusis *et al.*, 2012). Moreover, little evidence has been found so far that exposure to exogeneous hormones, like the use of hormonal contraception or hormonal replacement therapy in menopause, could increase

the risk of meningioma development (Custer *et al.*, 2006; Iplikcioglu *et al.*, 2014).

Other diseases thought to be associated with an increased risk of developing meningioma include pre-existing diabetes mellitus in both males and females between 40 and 69 years of age, and arterial hypertension, particularly in women aged 60-69 years (Schneider *et al.*, 2005; Nayeri *et al.*, 2016).

There is no clear correlation between meningioma and head trauma, but some studies suggest that patients with concussion, skull fracture or other head injuries followed up for 8 years have an increased standardized incidence ratio (SIR) for meningioma of 1.2 after the first year (Wiemels, Wrensch & Claus, 2010).

Mobile phone use has been shown to be unrelated to meninigoma incidence (Benson *et al.*, 2013).

1.6 The genetic landscape of meningioma

Losses of all or parts of chromosome 22 were first identified by fluorescent chromosome staining in meningioma patients (Mark, Levan & Mitelman, 1972; Mark, Mitelman & Levan, 1972). Moreover, monosomy 22 was also reported in people affected by meningioma in the context of the Neurofibromatosis type 2 (NF2) disorder (Rouleau *et al.*, 1993).

The tumour suppressor gene *NF2*, located on chromosome 22q12, was the first gene to be identified in the pathogenesis of meningioma (Fontaine *et al.*, 1991; Rouleau *et al.*, 1993; Trofatter *et al.*, 1993). It encodes for the protein

Neurofibromin 2 (also known as Merlin or Schwannomin), which belongs to the 4.1 family of cytoskeletal-associated proteins, and is involved in linking the plasma membrane to the actin cytoskeleton (Trofatter *et al.*, 1993).

Next-generation sequencing of meningioma tumour samples led to the identification of mutations in *TNF receptor-associated factor 7 (TRAF7), v-akt murine thymoma viral oncogene homolog 1 (AKT1), Krüpple-like factor 4 (KLF4)*, *Smoothened, frizzled family receptor (SMO)* (Clark *et al.*, 2013) and *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)* (Clark *et al.*, 2013; Abedalthagafi *et al.*, 2016) in non-*NF2* meningioma.

Other genes that have been observed to be involved in meningioma genesis are *SMARCB1* and *SMARCE1*, two subunits of the SWI/SNF chromatin remodeling complex (Smith *et al.*, 2013; Smith *et al.*, 2014), and *SUFU*, which is involved in the Shh-Gli1 pathway (Aavikko *et al.*, 2012).

Fig. 1.4 provides a schematic representation of the mutations so far identified in meningioma; mutations in *TRAF7, KLF4, AKT, PIK3CA*, and *SMO* are mutually exclusive of *NF2* mutations, whereas mutations in *TRAF7* can co-occur with mutations in *KLF4, AKT1*, and *PIK3CA* (Yuzawa, Nishihara & Tanaka, 2016).

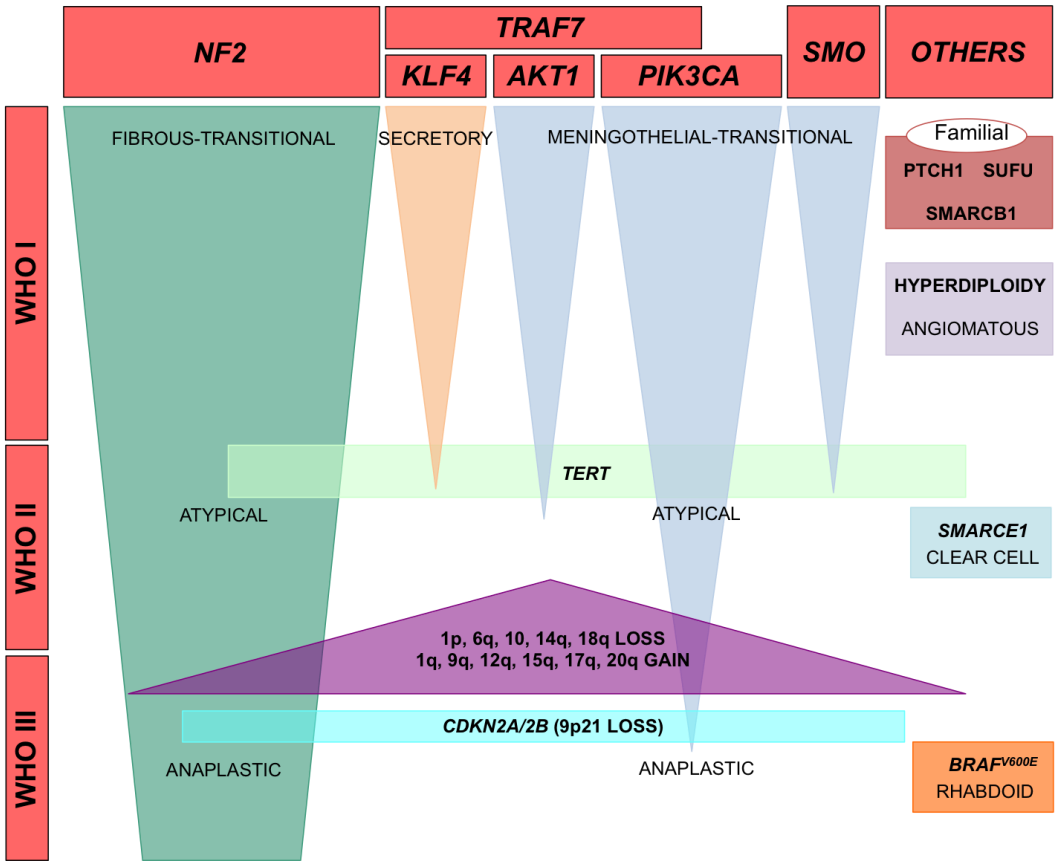


Figure 1.4 Schematic of genetic/cytogenetic alterations in meningioma. Mutations in *TRAF7, KLF4, AKT, PIK3CA*, and *SMO* are mutually exclusive of *NF2* mutations, whereas mutations in *TRAF7* can co-occur with mutations in *KLF4, AKT1*, and *PIK3CA.* Figure adapted from (Yuzawa, Nishihara & Tanaka, 2016).

1.6.1 *NF2*-associated meningiomas

Mutations of the *NF2* gene are the most commonly identified alterations in meningioma patients, and they can either be present as germline mutations or can be acquired somatically as a result of loss of heterozigosity (LOH) (Yuzawa, Nishihara & Tanaka, 2016).

Germline mutations in *NF2* are detectable in over 90% of all cases of nonmosaic NF2 disease and confer a significant risk of meningioma; it has been estimated that approximately 50% of people with NF2 will develop a meningioma during their lifetime. Moreover, the presence of intracranial meningiomas in NF2 disease is associated with the likelihood of disease mortality (Smith, 2015).

In the NF2 disease setting, it has been demonstrated that the type and location of the mutation in the gene correlates with the risk of developing a meningioma. Truncating mutations and mutations occurring toward the 5' end of the gene confer a greater risk of developing a meningioma compared to nontruncating mutations and mutations occurring in the 3' end of the gene (Smith *et al.,* 2011).

Somatic biallelic inactivation of *NF2* can be observed in almost two thirds of sporadic meningiomas. These sporadic tumours are thought to be less aggressive than those arising as a consequence of NF2 disease (Antinheimo *et al.*, 1997; Perry *et al.*, 2001). However, this difference could be due to a selection bias of the individuals referred for surgery (Goutagny *et al.*, 2012).

The mechanism by which Merlin drives meningioma genesis is still poorly understood. Merlin is a membrane-cytoskeleton linker involved in inhibition of cell proliferation through contact-dependent regulation of a number of signalling pathways, including Hippo, Patched, and Notch pathway (Curto & McClatchey, 2008). It also negatively regulates mammalian target of rapamycin complex 1 (mTORC1) and positively regulates the kinase activity of mTORC2 (Ammoun & Hanemann, 2011; Zhou & Hanemann, 2012; Petrilli & Fernandez-Valle, 2016), suggesting a role for Merlin in the activation of the mammalian target of rapamycin (mTOR) pathway during tumourigenesis.

Interestingly, in other tumours with *NF2* alterations, such as serous ovarian cancer and malignant pleural mesothelioma (MPM), increased sensitivity to focal adhesion kinase (FAK) ihibitors has been demonstrated, which may represent a therapeutic opportunity for meningiomas carrying *NF2* aberrations (Shah *et al.*, 2014; Shapiro *et al.*, 2014).

1.6.2 Mutations associated with non-*NF2* meningioma

TRAF7

TRAF7 is a proapoptotic N-terminal RING and zinc finger domain protein with E3 ubiquitin ligase activity; it potentiates MEKK3-mediated signalling and is involved in NF-κB signalling regulation (Bouwmeester *et al.*, 2004).

Mutations of *TRAF7* have been identified in nearly 25% of meningiomas, and they mostly occur in the WD40 domains of the protein, through which it binds various molecules to alter signalling through different pathways (Yuzawa, Nishihara & Tanaka, 2016).

AKT1

AKT1 is a protein kinase located downstream of the PI3K signalling pathway, and is involved in the regulation of cell survival and cell cycle progression (Porta, Paglino & Mosca, 2014).

AKT1 mutations occur in 5-8% of meningioma tumours (Clark *et al.*, 2013). All the mutations identified in *AKT1* were c.49G>A (p.E17K), which has also been identified in other cancers such as breast, colorectal, lung, bladder, ovarian, and endometrial carcinoma (reviewed in Yuzawa, Nishihara & Tanaka, 2016). This

mutation causes constitutive protein acitvation and promotes proliferation and tumour growth (Carpten *et al.*, 2007; Beaver *et al.,* 2013).

PIK3CA

The *PIK3CA* gene encodes for the catalitic subunit of PI3Ks (phosphoinositide 3 kinases), which are important upstream activators of many key signalling pathways, including the PI3K/AKT/mTOR pathway, crucial for the development and progression of several tumours (Alqahtani, Ayesh & Halawani, 2019).

The most frequent mutation in *PIK3CA* observed in meningioma is H1047R (42%). R108H, E110del, N345K, HC419del, E453K, and T1025T mutations were also observed in one case each out of 200 meningiomas analysed (Yuzawa, Nishihara & Tanaka, 2016). Amongst these mutations, H1047R and E545K lead to constitutive AKT and MEK1/2 phosphorylation, stimulation of the PI3K/AKT/mTOR pathway, growth factor-independent cell survival and confer resistance to PI3K inhibitors (Carpten *et al.*, 2007).

KLF4

KLF4 belongs to a family of DNA-binding transcriptional regulators involved in the regulation of proliferation, differentiation, migration, inflammation and pluripotency (Takahashi & Yamanaka, 2006; Tetreault *et al.,* 2013). All of the *KLF4* mutations observed in meningioma were c.1225A>C (p.K409Q); this aberration seems to be specific for meningioma, with only a few cases carrying the same alteration identified in other tumours. The mutated residue lies within the first zinc finger domain of the protein and makes DNA contact (Clark *et al.*, 2013; Yuzawa, Nishihara & Tanaka, 2016). *KLF4* mutations were identified in 11.8% tumours from a 93 skull base meningioma cohort (Yesiloz *et al.*, 2017).

SMO

Mutations in SMO, a G protein-coupled receptor involved in signal transduction of the hedgehog pathway (Endoh-Yamagami *et al.*, 2009), have been found in 5% of non-*NF2* tumours. Two mutations commonly affect this gene in meningioma: L412F and W535L (Yuzawa, Nishihara & Tanaka, 2016). The former is a known hotspot of basal cell carcinoma of the skin, whereas the latter is uncommon in other tumours, with a few cases reported only in medulloblastoma (Yuzawa, Nishihara & Tanaka, 2016).

Rare mutations in *SMO*, such as R113Q, L522V, and P647S, were identified concomitantly with the *NF2* mutation, suggesting that in these cases mutant *SMO* may not contribute to tumourigenesis (Yuzawa, Nishihara & Tanaka, 2016).

PTCH1 and SUFU

Germline mutations and second hit mutations or loss of heterozigosity were reported in meningioma for *PTCH1* and *SUFU* (Aavikko *et al.*, 2012; Kijima *et al.*, 2012).

PTCH1 is thought to hold SMO in an inactive state, thus inhibiting signalling to downstream genes (Wicking, Smyth & Bale, 1999; Tukachinsky *et al.*, 2016), whilst SUFU is the major negative regulator of the sonic hedgehog signalling, located downstream of SMO. It has been shown that a *SUFU* missense mutation, c.367C>T (p.Arg123Cys), is associated with inherited multiple meningioma disease in a single large family with five affected siblings (Aavikko *et al.*, 2012). However, a wider screening involving 121 individuals did not

identify any further germline mutations; this observation suggests that mutations in *SUFU* are an infrequent cause of meningioma (Clark *et al*., 2013).

These two genes are thought to share the same oncogenic pathway as *SMO* type meningioma (Smith *et al.*, 2014).

POLR2A

The *POLR2A* gene encodes for the catalytic subunit of the RNA polymerase II, which mediates the transcription of all protein-coding genes in eukaryotic cells. A study by Clark *et al.* aiming to identify oncogenic alterations in meningiomas found recurrent somatic mutations in *POLR2A* in ~6% of 775 tumours examined; interestingly, *POLR2A* mutations did not co-exist with any of the other mutations and tended to be associated with meningothelial histology and tumour location in the tuberculum sellae (Clark *et al.*, 2016). No *POLR2A* mutations were identified in high-grade meningiomas.

SMARCB1 and *SMARCE1*

SMARCB1 and SMARCE1 are subunits of the SWI/SNF ATP-dependent chromatin remodeling complex, which modulates chromatin architecture to enable DNA accessibility and gene expression (Clapier & Cairns, 2009), and serves critical roles in cells division, cell and tissue differentiation, and development (Kadoch & Crabtree, 2015).

Somatic *SMARCB1* mutations have been identified in rare cases of sporadic meningioma (Schmitz *et al.*, 2001). It has also been shown that people affected by schwannomatosis disease, characterised by germline mutations in *SMARCB1*, have a 5% increased risk of developing one or more meningiomas (Smith *et al.*, 2012).

NF2 and *SMARCB1* are both located on chromosome 22q, and somatic loss of most, if not all, of the long arm of chromosome 22 during tumour development means that they are frequently co-deleted. *SMARCE1* mutations appear to be specific for the clear cell histological subtype, which is classified as WHO grade II due to its apparent high recurrence rate. However, the recurrence rate observed in the small number of *SMARCE1*-related clear cell meningiomas reported so far appears to be low (Smith *et al.*, 2014).

Almost all of the mutations identified to date in *SMARCE1* are truncating mutations predicted to result in complete loss of the protein. They have also all occurred between exons 5 and 9, which encompass the high molecular weight group (HMG) domain and a conserved NHRLI (Asn-His-Arg-Leu-Ile) domain. It is possible that these regions may be important for the expression and/or structural integrity of the protein (Smith, 2015).

The extent of the role played by SMARCB1 and SMARCE1 in gene regulation and the mechanism by which they are involved in meningioma require further investigation.

DAL-1

DAL-1 (also known as Erythrocyte Membrane Protein Band 4.1 Like 3, EPB41L3) is a tumour suppressor protein that inhibits cell proliferation and promotes apoptosis (Wang *et al.*, 2014b).

A somatic mutation corresponding to loss of DAL-1 has been observed in meningioma, often in conjunction with *NF2* alterations (Gutmann *et al.*, 2000; Nunes *et al.*, 2005). *DAL-1* knockout mice have been used to investigate DAL-1 contribution to meningioma genesis and show no differences in cell proliferation

or growth characteristics and no increased risk of cancer (Yi *et al.*, 2005). This indicates that *DAL-1* loss is not an initiating event in meningioma growth but it instead occurs as a secondary effect during tumour development in *NF2*-associated meningiomas (Yi *et al.*, 2005).

1.6.3 Cytogenetic alterations associated with malignancy

Various cytogenetic alterations have been previously described in meningioma, usually in association with disease progression and increased malignancy. Loss of chromosomes 1p, 6q, 10p, 10q, 14q, and 18q are frequently observed in atypical and anaplastic meningiomas (Bello *et al.*, 1994; Simon *et al.*, 1995; Weber *et al.*, 1997; Lamszus *et al.*, 1999; Cai *et al.*, 2001; Aizer *et al.*, 2016).

Polysomy of chromosome 5, 13 and 20 was also described in angiomatous meningioma (Abedalthagafi *et al.*, 2014), but it appears to be not specific to this subtype. Gains of chromosome 5, 21q, 17q, 20q, 1q, 9q, and 15q were also common in high grade meninigoma tumours (Linsler *et al.*, 2014; Abedalthagafi *et al.*, 2014; Ketter *et al.*, 2007).

Progression to malignant meningioma is also associated with loss of *CDKN2A* ($p16^{INKa}$/$p14^{ARF}$), and *CDKN2B* ($p15^{INK4b}$), which are both located on chromosome 9p21. The alterations affecting these two genes include homozygous deletions, mutations, and lack of expression (Bostrom *et al.*, 2001; Yuzawa, Nishihara & Tanaka, 2016).

Other mutations correlating with increased tumour malignancy have been identified in the *Telomerase Reverse Transcriptase (TERT)* promoter which

have been shown to be significantly associated with shorter time to progression (Sahm *et al.*, 2016); the hotspots were C228T and C250T, which generate *de novo* consensus binding motifs for E-twenty-six (ETS) transcription factors, leading to increased transcriptional activation of *TERT* (Goutagny *et al.*, 2014). These mutations have been observed to occur with or without *NF2* loss/mutations (Goutagny *et al.*, 2014).

1.7 Current treatment options for meningioma

The general consensus for the management of small incidental meningiomas is to actively monitor the patients by clinical and radiological follow-up. Usually, treatment is withheld until symptoms develop, sustained growth occurs or concerns of encroachment on sensitive structures arise (Rogers *et al.*, 2015).

First-line treatments for meningioma include surgery, which can be followed in selected cases by fractionated radiation therapy (RT) or stereotactic radiosurgery (SRS). Indications for treatment depend on factors such as age, comorbidity, life expectancy, patient preference, histological grade, molecular factors, tumour location, and extent of resection (Brastianos *et al.*, 2019).

Multimodality treatment is required in cases of clinically aggressive meningiomas with early and multiple recurrences; in selected cases patients are referred for chemotherapy or experimental clinical trials for treatment-refractory tumours. However, so far no effective chemotherapeutic treatment has been found (Wen *et al.*, 2010).

1.7.1 Surgery for intracranial meningioma

Surgery is the primary treatment for the majority of patients with symptomatic and enlarging meningiomas (Goldbrunner *et al.*, 2016), as it not only removes the tumour to relieve mass effect, but it can also rapidly improve the neurological functions and control seizures (Englot *et al.*, 2016; Chen *et al.*, 2017b).

The gold standard for meningioma is complete resection of the tumour and any involved dura or bone.

Since the recognition that meningiomas are able to regrow despite satisfactory surgery, in 1957 Simpson described a classification system to define the risk of recurrence following surgery alone based on the surgeon's intraoperative assessment of the extent of resection (Table 1.1) (Simpson, 1957). As the extent of resection is the main factor involved in determining the risk of recurrence, neurosurgeons aim for maximum safe resection with low morbidity and preservation of neurological function (Di Maio *et al.*, 2012; Misra, 2012). Prediction of recurrence risk is based on extent of resection and tumour grade; recurrence risks at 10 years for WHO I, II and III meningioma are approximately 6%, 17% and 30%, respectively (Holleczek *et al.*, 2019).

Simpson grade	Definition	Extent of resection
I	Gross total resection of tumour, dural attachment, and abnormal bone	GTR
II	Gross total resection of tumour, coagulation of dural attachment	GTR
III	Gross total resection of tumour without resection or coagulation of dural attachments, or extradural extensions	GTR
IV	Partial tumour resection	STR
V	Tumour biopsy	STR

Table 1.1 Simpson grades of resection and corresponding EORTC/RTOG definitions of extent of resection. GTR = gross total resection, STR = subtotal resection (Simpson, 1957; Vogelbaum *et al.*, 2010).

Meningioma surgery can be challenging; attention must be paid to the venous anatomy in order to avoid postoperative venous infarction, which has devastating consequences for the patient (Brastianos *et al.*, 2019). Reconstruction of major venous sinuses remains controversial as there is a 3% risk of death and a 21% risk of major morbidity (Sindou & Alvernia, 2006; Han *et al.*, 2016a). The alternative is to leave meningioma invading the sinus, and subsequently monitor it or treat with adjuvant RT or SRS (Combs *et al.*, 2012), although it is preferable to remove the tumour completely where possible.

Image guidance is routinely used in cranial neurosurgery. Intraoperative MRI (iMRI) and CT (iCT) can be used to update neuronavigation and identify residual tumour (Yamashita *et al.*, 2013; Terpolilli *et al.*, 2016; Schichor *et al.*, 2017). By using a somatostatin receptor agonist, bound with a DOTA bifunctional chelator and a radionuclide, such as gallium-68 or lutetium-177 (Fani, Nicolas & Wild, 2017), DOTATATE PET can be used to discriminate between meningioma and normal surrounding tissue with greater sensitivity than MRI (Fani, Nicolas & Wild, 2017; Rachinger *et al.*, 2015) and it can also be used as a predictor of tumour growth rate (Rachinger *et al.*, 2015).

Intraoperative neurophysiological monitoring may help minimize postoperative neurological deficits in critically located lesions (Brastianos *et al.*, 2019).

Studies of surgical adjuncts such as 5-aminolevulinic acid (5-ALA) fluorescence have shown heterogeneous fluorescence within meningioma as well as nonspecific fluorescence in adjacent brain (Motekallemi *et al.*, 2015), suggesting a limited role for this compound in surgical approaches for this type of tumour. However, a recent review found that 5-ALA may be useful in high grade meningioma patients (Motekallemi *et al.*, 2015).

Patients with recurrent meningioma represent a surgical challenge as surgery success may be hampered by scar tissue, and the underlying brain is often more friable and susceptible to injury. Moreover, the scalp may have a reduced ability to heal appropriately in case of repetitive surgery (Brastianos *et al.*, 2019).

1.7.2 Radiation treatment

Radiation therapy is the only non-surgical standard of care for the management of meningioma (Goldbrunner *et al.*, 2016).

Adjuvant radiotherapy is often used in atypical, malignant or recurrent meningioma, when total resection is not possible. However, we still lack appropriate trials to define the most suited patients, tumour target volumes, radiation doses and fractionation schemes, especially for atypical meningiomas (Rogers *et al.*, 2015). For these patients, an EORTC trial is ongoing, comparing the results of observation versus radiation therapy following tumour resection (Jenkinson *et al.*, 2015).

Despite these problems, excellent local control has been observed following various forms of RT, including fractionated external beam radiation therapy (EBRT), SRS, and brachytherapy (Brastianos *et al.*, 2019).

1.7.3 Systemic treatments

Systemic therapies may be useful for patients who present with progression after failure of all other lines of treatment, and in the rare case of metastatic meningiomas (Mawrin, Chung & Preusser, 2015).

However, currently available chemotherapies have proven to be ineffective in meningioma patients (Chamberlain, 2012; Rogers *et al.*, 2015), or met with significant toxicity (Norden *et al.*, 2010; Kaley *et al.*, 2015).

Hydroxiurea (HU) is used as a chemotherapeutic treatment in patients with surgically and radiation refractory meningiomas, but a retrospective case study of 35 patients showed that, although HU is well tolerated, it had very limited activity, indicating the need for alternative treatments for recurrent high grade meningiomas (Chamberlain, 2012).

1.7.3.1 Molecular therapy

The recent identification of specific genetic alterations in meningioma has opened up potential targeted treatment options for affected patients, some of which are outlined below (Fig. 1.5).

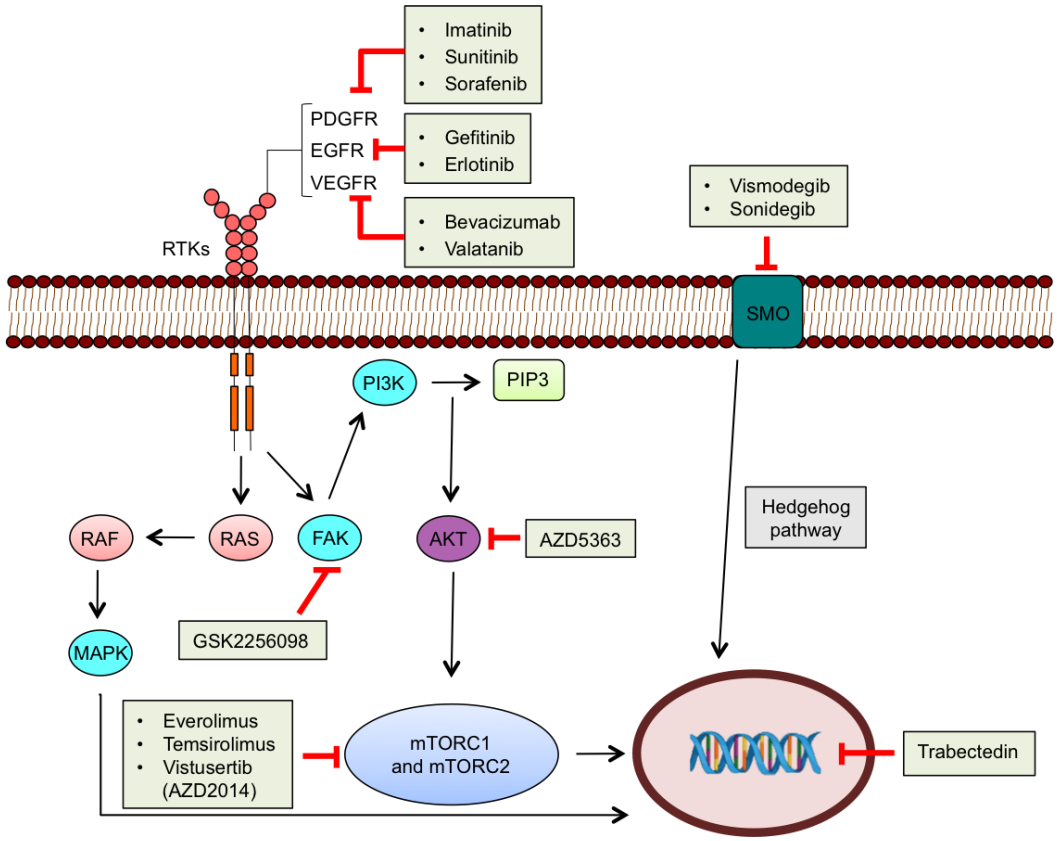


Figure 1.5 Overview of activated signalling pathways and drug targets in meningioma. The figure provides a summary of the current medical treatment

options for meningioma. Some of the available agents are employed for their effects on molecular targets that could represent biomarkers for patient selection. Figure adapted from (Preusser, Brastianos & Mawrin, 2018).

A phase II trail with Sunitinib malate (SUO11248), an orally administered tyrosine kinase inhibitor targeting VEGF receptor (VEGFR), PDGFR, and KIT, met the primary efficacy endpoint, with progression free survival at 6 months (PFS6) of 42% in the primary cohort of atypical and anaplastic meningioma patients. However, despite apparent efficacy, considerable toxicity was observed (Kaley *et al.*, 2015), but the authors warrant further studies in a randomized setting.

A randomized, multicentre phase II trial for patients with recurrent WHO II/III meningiomas (EORTC-1320-BTG) has recently completed accrual to test the efficacy of Trabectedin, a chemotherapeutic agent routinely used for advanced sarcoma and ovarian cancer (Demetri *et al.*, 2009; Kaye *et al.*, 2011). Trabectedin has shown efficacy in *in vitro* studies in meningioma, however the outcome of the trial was negative as Trabectedin did not improve PFS and overall survival (OS) and was associated with significantly higher toxicity compared to the local standard of care (Preusser *et al.*, 2012).

As briefly mentioned above, Merlin is involved in the activation of the mTOR pathway during the pathogenesis of meningioma, by negatively regulating mTORC1 and positively regulating mTORC2 (James *et al.*, 2009; James *et al.*, 2012). Thus, overexpression of mTORC1 pathway components has been observed in meningioma, and mTORC1 inhibitors, such as Everolimus and Temsirolimus, are able to suppress meningioma growth in preclinical mouse

models (Pachow *et al.*, 2013). Everolimus has been used in a phase II study in combination with Bevacizumab, leading to stable disease for more than 6 months in 35% of patients (Shih *et al.*, 2016). Moreover, the dual mTORC1 and mTORC2 inhibitor Vistusertib (AZD2014) showed promising results in preclinical studies in blocking proliferation of meningioma cells (Beauchamp *et al.*, 2015), and is now under investigation in a phase II trial in patients with recurrent WHO II and III meningiomas (NCT03071874), and in NF2 patients with either progressive or symptomatic meningiomas (NCT02831257) (Brastianos *et al.*, 2019). However, in this latter group, the clinical trial did not reach its endpoints, showing that AZD2014 is not an effective treatment for NF2 patients (personal communication).

FAK inhibition may be another potential strategy for *NF2*-mutant meningiomas, as other tumours carrying *NF2* alterations are shown to be sensitive to FAK inhibition in *in vivo* studies (Shah *et al.*, 2014; Shapiro *et al.*, 2014). FAK inhibitors in *NF2*-altered meningiomas are currently being investigated in a national Alliance-sponsored phase II trial (NCT02523014/A071410) (Brastianos *et al.*, 2019).

Anti-angiogenic inhibitors have shown limited efficacy in meningiomas. Bevacizumab showed some anti-neoplastic activity in retrospective studies, but these results need to be further validated in randomized controlled trials (Lou *et al.*, 2012; Nunes *et al.,* 2013). Vatalanib and Sunitinib have shown limited efficacy in prospective studies, with response rates of 0% and 6%, respectively (Raizer *et al.*, 2014; Kaley *et al.*, 2015).
It was recently shown that meningiomas with higher levels of DNA methylation in specific CpG islands are more likely to recur (Sahm *et al.*, 2017; Olar *et al.*,

2017; Vasudevan *et al.*, 2018); thus, compounds targeting epigenetic modifiers warrant further investigation in meningioma (Tumber *et al.*, 2017; Olar *et al.*, 2017).

As the PI3K/AKT/mTOR and the Hedgehog (Hh) pathways are found to be aberrantly activated in meningioma, molecular agents targeting them may represent promising approaches. AKT inhibitors are showing promising results in other *AKT*-mutated tumours (Hyman *et al.*, 2017). A recent case report showed promising response to an AKT inhibitor (AZD5363) in a patient with multiple, recurrent intracranial meningiomas (Weller *et al.*, 2017) who, after one year of treatment with AZD5363, had a sustained significant clinical and radiographic response to the drug (Weller *et al.*, 2017). Hh pathway inhibitors have not been tested in meningioma patients to date, but two Hh inhibitors (Vismodegib and Sonidegib) are already FDA approved for the treatment of basal cell carcinoma (Silapunt, Chen & Migden, 2016). A phase II trial in the US is currently investigating the efficacy of SMO, AKT1, and FAK inhibitors in patients with residual, recurrent, or progressive meningiomas harbouring targetable alterations in *SMO*, *AKT1*, and *NF2*, respectively (NCT02523014/A071401) (Brastianos *et al.*, 2019).

Other potential genetic driver alterations in non-*NF2* meningiomas include mutations in *CDKN2A* and *CDKN2B*, which are also frequently linked to malignant progression. Thus, Cyclin-dependent kinase pathway inhibition might be a potential target to treat high-grade meningiomas and should be further investigated in preclinical and clinical studies (Bostrom *et al.*, 2001; Goutagny *et al.*, 2010).

1.7.3.2 Immunotherapy

Even though fewer somatic targetable alterations have been observed in WHO III compared to WHO I meningiomas, these tumours appear to harbour mutations predicted to be neoantigens, making them suitable candidates for immunotherapy (Bi *et al.*, 2017).

Recent observations that expression of programmed death ligand 1 (PD-L1) was increased in malignant meningioma (Du *et al.*, 2015b; Han *et al.*, 2016b), and that a higher number of PD-L1 expressing cells was associated with worse overall survival (Han *et al.*, 2016b), further support this hypothesis.

PD-L1 is commonly expressed on tumour and/or immune cells in the tumour microenvironment and interacts with programmed death 1 (PD-1) on tumour infiltrating lymphocytes, attenuating effector T cell responses and allowing tumours to escape immune attack (Chen *et al.*, 2019). Antibody-mediated PD-L1 blockade has shown clinical benefit in locally advanced or metastatic cancer of different types (Powles *et al.*, 2014; Massard *et al.*, 2016; Balar *et al.*, 2017).

In a recently published study, authors tested the efficacy of the monoclonal antibody Avelumab on patient-derived meningioma cell lines that constitutively express high levels of PD-L1 and found that it could direct meningioma cell lysis by healthy donor natural killer (NK) cells through antibody-dependent cellular toxicity (ADCC). The authors translated their findings into an *in vivo* orthotopic skull-based model, giving promising evidence that immunotherapy using Avelumab-directed ADCC against meningioma may offer a novel treatment approach (Giles *et al.*, 2019).

Furthermore, there is proof that meningioma contains antigen-experienced effector T cells of an exhausted phenotype (Fang *et al.,* 2013), suggesting that an immunosuppressive microenvironment may be involved in the malignant phenotype of these tumours (Du *et al.*, 2015b).

Phase II trials are currently underway to investigate the efficacy of the immune checkpoint inhibitors Nivolumab and Pembrolizumab in recurrent or residual high-grade meningiomas (NCT03279692, NCT02648997). Combination therapy consisting of RT and checkpoint blockade may be beneficial to meningioma patients, as has been shown for other neoplasms (Sharabi *et al.*, 2015).

1.8 Issues regarding meningioma classification

Despite the genetic landscape of meningioma having been well established over the years, those findings have not all been thoroughly tested for their prognostic relevance (Sahm *et al.*, 2017). Moreover, meningioma grading is still based solely on the histopathological observation of tumour biopsies, which is subjected to inter-observer variability (Willis *et al.*, 2005), and the method has been questioned due to the little prognostic effect of the histological criteria (Rogers *et al.*, 2016; Vaubel *et al.*, 2016; Baumgarten *et al.*, 2016). In this section, I will discuss some of the efforts made recently in order to overcome the issues affecting meningioma grading and towards the definition of reliable and more objective molecular stratification markers.

1.8.1 Identification of methylation classes for meningioma prognosis

A recent large, multicentre study investigated the genome-wide DNA methylation patterns in meningiomas from ten European academic neuro-oncology centres to identify distinct methylation classes in meningioma tumours (Sahm *et al.*, 2017), aiming to define biologically and clinically relevant subgroups.

This study also included samples of other extra-axial skull tumours that might histologically mimic meningioma variants and arise in or infiltrate the meninges, in order to understand whether these tumours could be separated from meningiomas on the basis of DNA methylation.

Indeed, this method proved effective in distinguishing between meningiomas and other extra-axial skull tumours, and identified two major epigenetic groups in meningioma, group A and B, shown in Fig. 1.6.

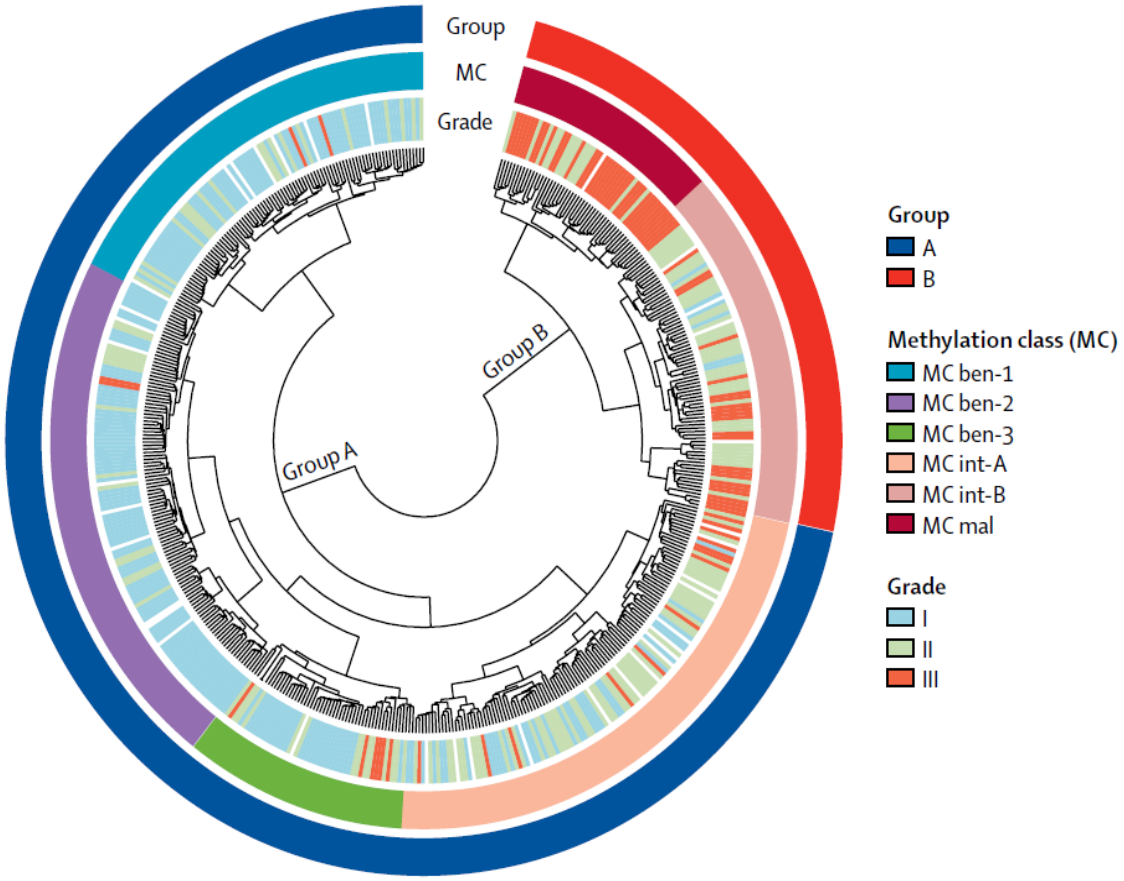


Figure 1.6 Unsupervised clustering of 497 meningioma samples considered in the study. Two major methylation groups have been observed, group A and B, with a further subdivision into four subgroups in group A (MC ben-1, MC ben-2, MC ben-3, and MC int-A) and two subgroups in group B (MC int-B and MC mal). MC ben = methylation class benign, MC int = methylation class intermediate, MC mal = methylation class malignant (Sahm *et al.*, 2017). Figure adapted from (Sahm *et al.*, 2017).

Interestingly, the clustering of meningioma samples into methylation classes (MC) was independent of grading. In fact, there was an enrichment of grade I tumours in the three benign methylation classes and an enrichment of WHO III tumours in the malignant methylation class, whereas WHO II tumours were

scattered across all methylation classes (Fig 1.6). Furthermore, tumours clustering in the same methylation class shared similar features regarding location, mutations, cytogenetic alterations, and progression-free survival (Fig. 1.7). Remarkably, stratification for methylation class was of higher value for prediction of progression-free survival than the WHO grading (Sahm *et al.*, 2017).

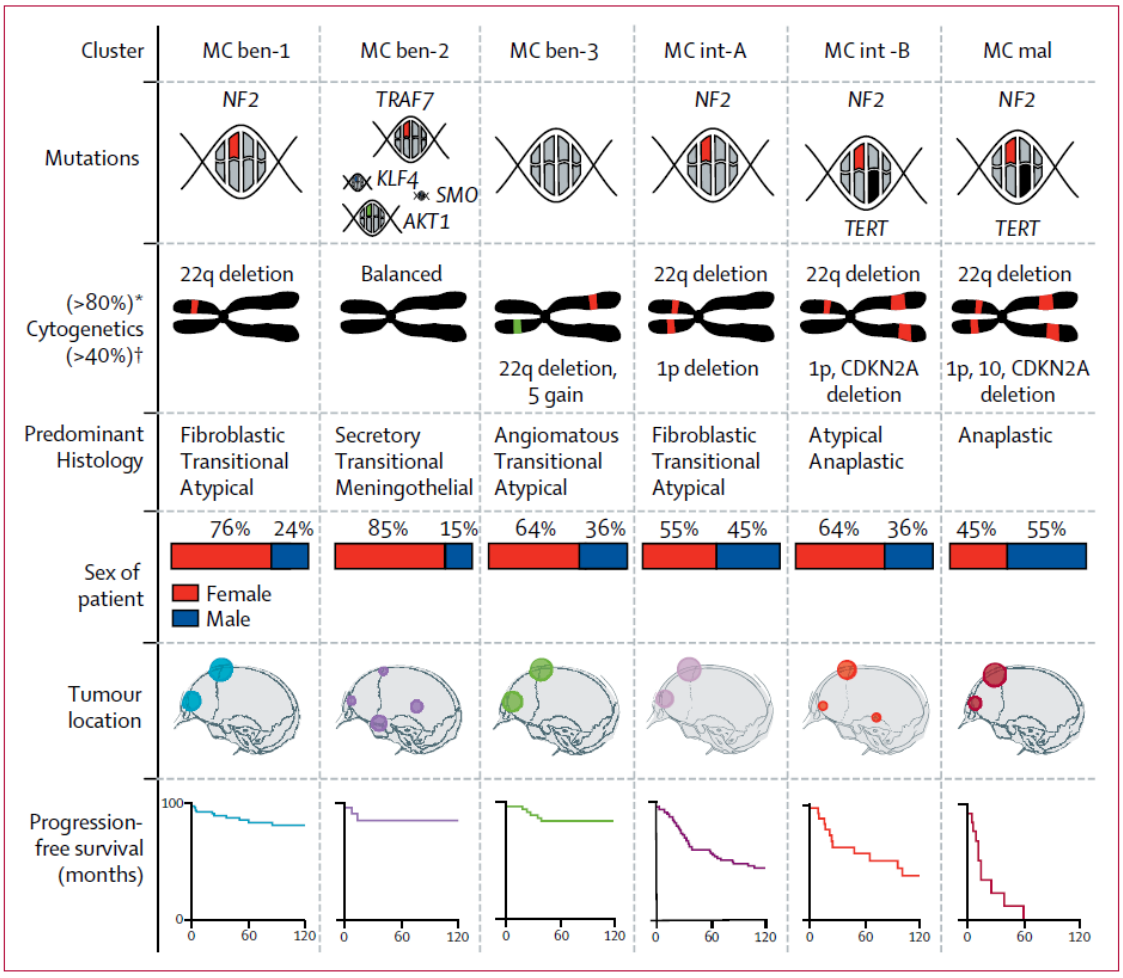


Figure 1.7 Overview of the six methylation classes and their molecular and clinical characteristics. Age distribution was similar throughout all methylation classes, but a predominance of male patients was observed in MC mal, while all other methylation classes comprised mainly of female patients.

*More than 80% of cases affected by the aberration, † More than 40% of cases affected by the aberration (Sahm *et al.*, 2017). Figure from (Sahm *et al.*, 2017).

The method for the identification of methylation classes in meningiomas used in this study corresponds to the present approach for other tumours, such as ependymoma and medulloblastoma, for which methylation profiling proved to be more relevant than histological grading (Pajtler *et al.*, 2015; Louis *et al.*, 2016). This has prompted the WHO to suggest an integrated reporting of both histological and molecular data (Sahm *et al.*, 2017).

As tumours clustering in the same methylation class tend to share the same genetic alterations, this method can also inform about the probability of therapeutic target availability, especially important for tumours arising in locations in which surgical resection is challenging, such as the skull base (Sahm *et al.*, 2017).

1.8.2 Identification of Cyclin D1 as a potential biomarker for meningioma

It has long been known that proliferative activity has a prognostic significance in a large variety of human solid tumours (Daidone, Costa & Silvestrini, 2001) and that many pathways involved in cell cycle control, such as components of the p16/CDK/Cyclin D1/RB pathway (Fig. 1.8), contribute to brain tumour development (Perry, Gutmann & Reifenberger, 2004). Furthermore, Cyclin D1 has been shown to be upregulated early on in many human cancers, suggesting its contribution to the development of the malignancies (Fu *et al.*,

2004) through induction of gene transcription, tumour growth, inhibition of apoptosis and induction of resistance to chemotherapy (Baldin *et al.*, 1993; Yu, Geng & Sicinski, 2001; Gao *et al.*, 2004; Wang *et al.*, 2012; Ravikumar & Ananthamurthy, 2014).

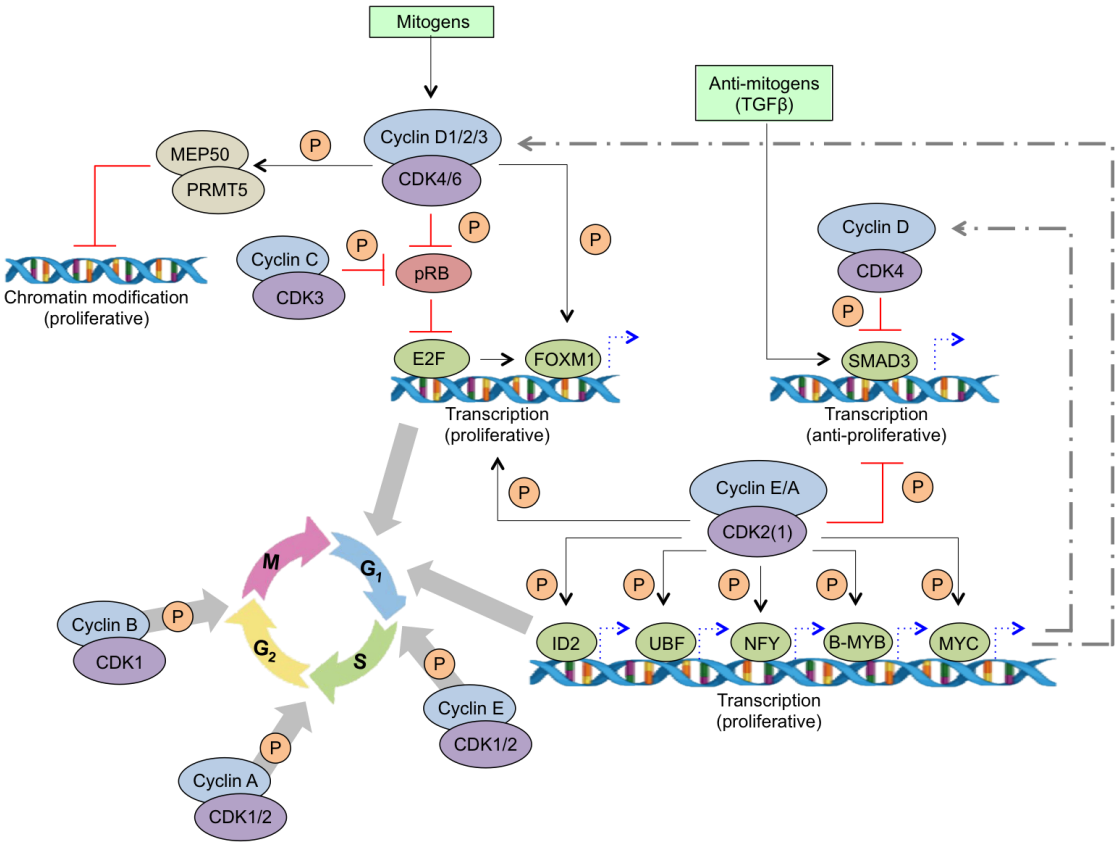


Figure 1.8 Roles of Cyclins and Cyclin-dependent kinases in the cell division cycle. Once growth factors induce the expression of D type Cyclins (Cyclins D1, D2 and D3), they interact with Cyclin-dependent kinase (CDK) 4 or CDK6 and phosphorylate the pocket proteins pRB, p107 and p130, which bind to and regulate E2F transcription factors during the G_1 phase of the cell cycle. In late G_1, E-type Cyclins (Cyclins E1 and E2) become upregulated and interact with CDK2, resulting in phosphorylation of a broader range of cell cycle related proteins and allowing progression to the S phase. The subsequent induction of

Cyclin A2 and B1 drive the progression of cells through the remainder of the cell cycle. Figure adapted from (Hydbring, Malumbres & Sicinski, 2016).

However, Cyclin D1 expression has only recently been studied in meningioma. Two studies in 2007 and 2008 showed that Cyclin D1 is overexpressed in meningioma tumour specimens compared to healthy tissue, and that it was present at higher levels in tumours with a higher proliferative index, assessed by proliferating cell nuclear antigen (PCNA) and MIB-1 levels (Alama *et al.*, 2007; Milenkovic *et al.*, 2008). More recently, it has been shown that Cyclin D1 expression correlates significantly with histological grade and recurrence rates (Cheng *et al.*, 2015).

Functional studies *in vitro* showed that Cyclin D1 knockdown caused an increase of the number of cells in G_0/G_1 and a reduction of those in the S phase of the cell cycle compared to control cells. Moreover, as a consequence of Cyclin D1 downregulation, the authors observed significantly reduced survival rates due to lower levels of the anti-apoptotic proteins Bcl-2 and survivin, and a decrease in cell invasion due to decreased levels of matrix metalloprotease-9 (MMP-9) (Cheng *et al.*, 2015). All together, these results suggest Cyclin D1 as a potential prognostic marker and an attractive therapeutic target for meningioma treatment.

1.8.3 microRNAs as potential meningioma biomarkers

Recently, a number of studies focussed their attention on microRNA expression levels in meningioma (Kliese *et al.*, 2013; Zhi *et al.*, 2013; Ludwig *et al.*, 2015; Wang *et al.*, 2015; Zhi *et al.*, 2016; El-Gewely *et al.*, 2016).

microRNAs (miRNAs) are small non-coding RNAs of around 22 nucleotides in length involved in the regulation of gene expression (Carthew & Sontheimer, 2009). They play a role in many physiological and pathological processes with the first evidence of their contribution to tumorigenesis arising in 2002, when miR-15a and -16-1 were found to be deregulated in chronic lymphocytic leukaemia (CLL), as they are located on chromosome 13q14, frequently deleted in CLL (Calin *et al.*, 2002).

1.8.3.1 miRNA biogenesis and function

miRNA-encoding genes are transcribed by the RNA polymerase II and transcripts are capped and polyadenylated to form the primary miRNA (pri-miRNA). In order to give rise to the active structure, the pri-miRNA has to undergo two processing steps. The first takes place in the nucleus and is carried out by an RNase III enzyme called Drosha, which cleaves the stem-loop structure to form the precursor-miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm by Exportin-5, where it is subjected to the second processing step by Dicer, which excises the terminal loop to create the mature miRNA duplex. The duplex is then unwounded by association with Argonaute (AGO) proteins and one of the strands is retained in order to form the RNA-

induced silencing complex (RISC), while the other is frequently degraded (summarised in Fig. 1.9) (Kim, 2005; Carthew & Sontheimer, 2009; Matsuyama & Suzuki, 2019).

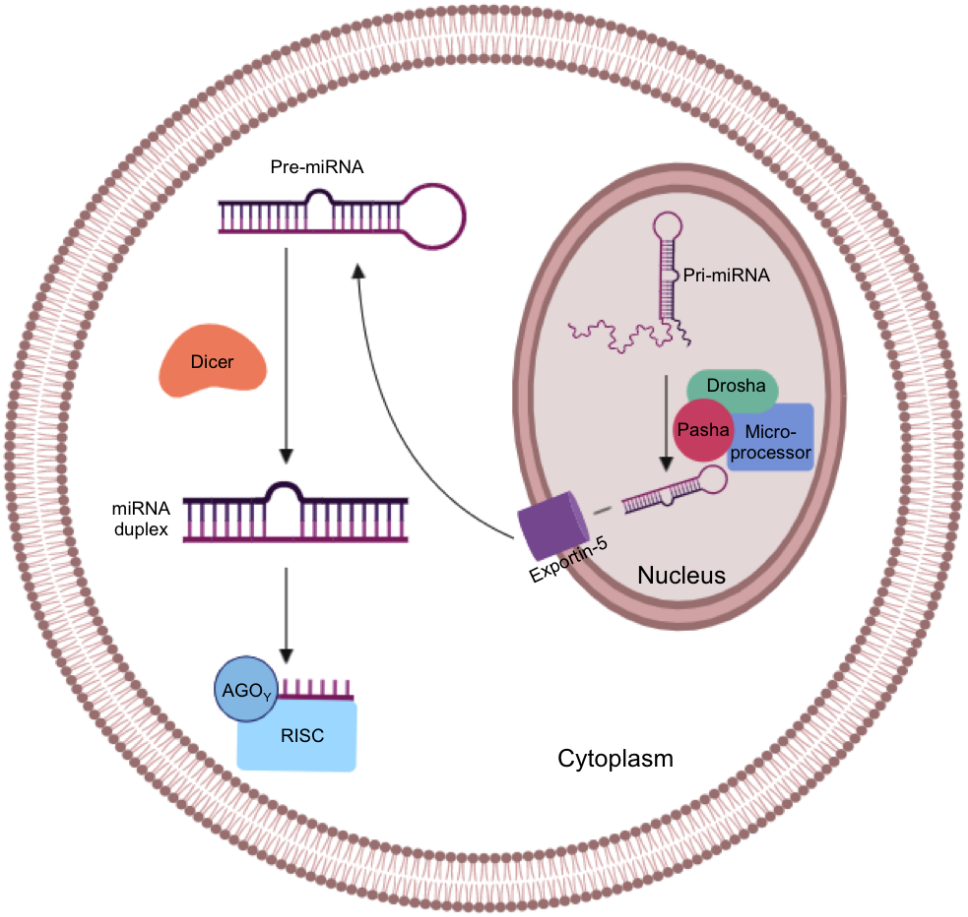


Figure 1.9 miRNA maturation and RISC assembly. Summary of the maturation process miRNAs are subjected to in order to form the active silencing complex: the pri-miRNA is cleaved in the nucleus by Drosha to form the pre-miRNA. This is then exported to the cytoplasm by Exportin-5, where Dicer excises the terminal loop to create the mature miRNA duplex. The duplex is then unwounded by association with Argonaute (AGO) proteins and one of the strands is retained in order to form the RNA-induced silencing complex (RISC), while the other is frequently degraded (Kim *et al.,* 2005; Carthew & Sontheimer, 2009). Figure adapted from (Tang, 2005).

RISC complexes can be distinguished as cleaving or non-cleaving RISCs, depending on the type of AGO protein recruited (Tang, 2005). In order to have a functional effect, both complexes need to bind to the miRNA binding sites on the target, which usually lay in the 3' UTR of the target messenger RNAs and are present in multiple copies (Carthew & Sontheimer, 2009).

A cleaving RISC can direct the target either for cleavage or translational repression (Hutvagner & Zamore, 2002; Tang, 2005), depending on the complementarity between the miRNA and the mRNA. If the base pairing is not extensive, the target may be physically unreachable by the endonuclease activity in the cleaving RISC, resulting in translational repression rather than efficient cleavage (Chiu & Rana, 2003; Haley & Zamore, 2004). Conversely, non-cleaving RISCs lack the slicer activity in the AGO protein and can direct the target only for translational repression.

1.8.3.2 miRNA studies in meningioma

As frequently observed in cancer, miRNAs are subjected to deregulation in meningioma as well, thus representing potential biomarkers, both in tissue and in the bloodstream.

With regards to studies carried out using meningioma tissues, in 2013 miR-145 was shown to be significantly downregulated in atypical and anaplastic meningioma tumours, and its reintroduction into a malignant meningioma cell line led to reduced proliferation, increased sensitivity to apoptosis, reduced anchorage-independent growth and reduction of orthotopic tumour growth in nude mice when compared to control cells. Moreover, malignant cells

expressing high levels of miR-145 had impaired migratory and invasive potential, both *in vitro* and *in vivo* (Kliese *et al.*, 2013).

In the same year, Zhi and colleagues screened the expression of 200 miRNAs in a training sample set and then validated significantly altered miRNAs in a secondary independent sample set; 14 miRNAs were found to be deregulated in meningioma samples compared to normal adjacent tissue samples (miR-17-5p, -22-3p, -24-3p, -26b-5p, -27a-3p, -27b-3p, -29c-3p, -96-5p, -146a-5p, -155-5p, -186-5p, -190a, -199a, and -219-5p). Of these 14 candidates, miR-29c-3p and -219-5p were found to be associated with advanced clinical stages of meningioma. Moreover, Kaplan-Meier analysis showed that high expression of miR-190a, paired with low expression of miR-29c-3p and -219-5p, correlated with higher meningioma recurrence rates. miR-190a expression level has also been shown as an important prognostic predictor independently of other clinicopathological factors, suggesting the use of miRNAs as prognostic tools for predicting tumour recurrence in meningioma (Zhi *et al.*, 2013).

An independent study identified 13 miRNAs differentially regulated between different subtypes of benign meningioma, and 52 miRNAs deregulated in anaplastic compared to benign meningioma. This study identified a 4-miRNA signature in tissues (miR-222, -34a*, -136, and -497) which showed promise to be used as a biomarker to differentiate between WHO II and I meningiomas (Ludwig *et al.*, 2015).

Other miRNAs studied in meningioma tissues include miR-200a, which was found to be deregulated in benign meningioma and whose overexpression inhibited tumour growth *in vitro* and *in vivo*, through regulation of β-catenin (Saydam *et al.*, 2009; Senol *et al.*, 2015). Furthermore, miR-224 has been

shown to target ERG2 and to contribute to malignant meningioma progression (Wang *et al.*, 2015), along with miR-218, -34a, -143, -193b, -451, and -21 (El-Gewely *et al.*, 2016).

It has recently been discovered that microRNAs are stably expressed in human blood, either as free circulating RNAs or carried in exosomes (Valadi *et al.*, 2007; Mitchell *et al.*, 2008; Lawrie *et al.*, 2008). This is particularly interesting since miRNA signatures in blood may serve as disease fingerprints and provide novel circulating biomarkers for cancer (Chen *et al.*, 2008; Mitchell *et al.*, 2008). Blood-based biomarkers have the important advantage of being minimally invasive, high-throughput and affordable to screen large groups of patients quickly (Zhi *et al.*, 2016).

In a study published in 2016, a serum 6-miRNA panel was suggested as a non-invasive biomarker for meningioma. Authors showed that serum levels of free circulating miR-106a-5p, -219-5p, -375, -409-3p, -197, and -224 could distinguish meningioma patients from healthy controls with high sensitivity and specificity. Moreover, this signature changed after tumour removal in meningioma patients, highlighting their usefulness to help monitor the effect of surgical resection in clinical practice. Furthermore, high levels of miR-409-3p, together with low levels of miR-224, correlated with higher recurrence rates in meningioma patients (Zhi *et al.*, 2016).

Although there are some limitations to the experimental design, such as failure to assess the miRNA signature in different ethnic populations and in meningioma patients who underwent adjuvant treatment, this study suggested that circulating miRNAs have potential to be used in diagnostic settings and as monitoring biomarkers for meningioma.

Another shortcoming of this study was that it only investigated the expression levels of miRNA extracted from whole serum. However, previous data shows that miRNAs in serum primarily exist inside exosomes and that this enriched fraction increases the sensitivity of miRNA detection (Gallo *et al.*, 2012).

1.9 Exosomes

Exosomes are extracellular vesicles (EVs) of 40-150 nm released by nearly all cell types (Thery, Zitvogel & Amigorena, 2002; Kahlert & Kalluri, 2013; Raposo & Stoorvogel, 2013).

They were first described by Pan and Johnstone in 1983 (Pan & Johnstone, 1983), when they observed that transferrin receptors were released into the extracellular space associated to a type of small vesicle during the maturation of sheep reticulocytes (Pan & Johnstone, 1983; Pan & Johnstone, 1984).

To date a number of different extracellular vesicles has been described (Cocucci, Racchetti & Meldolesi, 2009) but over the past three decades no unified terminology has been suggested (Zhang *et al.*, 2015). Currently, EVs can be grouped into two general classes, according to the way they are released from the cells. One of these classes encompasses microvesicles, which are directly shed from the cell membrane, while the second one comprises exosomes, which are released by exocytosis by multivesicular bodies (MVBs) fused with the plasma membrane (Fig 1.10) (Heijnen *et al.*, 1999; Bebelman *et al.*, 2018).

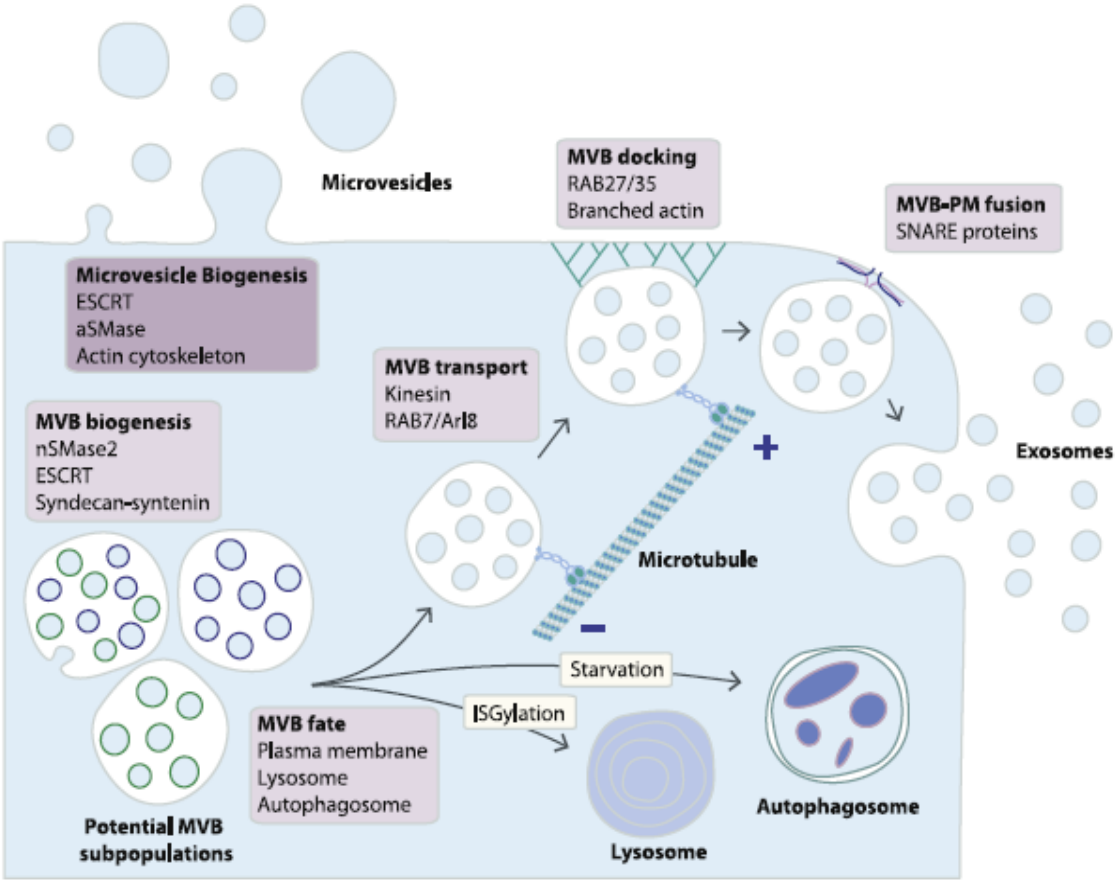


Figure 1.10 Schematic representation of exosome and microvesicle biogenesis pathways. Microvesicles bud directly from the plasma membrane, while exosomes are generated within multivesicular bodies (MVB) subpopulations. MVB pathways include fusion with lysosomes or autophagosomes, but little is known about the mechanism determining their fate. The process leading to MVB fusion with the plasma membrane is tightly regulated and includes trafficking of the MVBs along microtubules, docking at the plasma membrane and SNARE-mediated fusion. Figure from (Bebelman *et al.*, 2018).

Exosomes are first formed by invagination of the plasma membrane to form intracellular endosomes. During their maturation, inward budding of the endosomes leads to accumulation of intraluminal vesicles (ILVs), sequestering proteins, lipids, and nucleic acids, including mRNAs, miRNAs, and other non-coding RNAs (ncRNAs) in their lumen (Sato-Kuwabara *et al.*, 2015). These structures are referred to as multivesicular bodies or MVBs (Colombo, Raposo & Thery, 2014).

In most cells, the main fate of MVBs is to fuse with lysosomes, ensuring the degradation of their content; alternatively, they can fuse with the plasma membrane to release their cargo into the extracellular *milieu* (Jaiswal, Andrews & Simon, 2002).

The precise function of exosomes is still unknown; early hypothesis favoured the notion that exosomes could be cellular “garbage bags”, used to expel excess and/or non-functional cellular constituents (Thery, Zitvogel & Amigorena, 2002). However, since they are endocytic in origin, they could also be involved in the recycling of cell surface receptors, thus modulating outside-in signalling (Pan *et al.*, 1985; Thery, Zitvogel & Amigorena, 2002). More recently, it has been discovered that exosome contents can be transferred to a recipient cell to mediate phenotypic alterations, supporting the notion that exosomes are dynamic mediators of intercellular communication (Valadi *et al.*, 2007; Zomer *et al.*, 2015).

There are different mechanisms of interaction between exosomes and recipient cells; the transmembrane proteins of exosomes can interact with signalling receptors of the recipient cells (Munich *et al.*, 2012), or exosomes can fuse with the plasma membrane of the target cells and deliver their content into the

cytosol (Mulcahy, Pink & Carter, 2014). Furthermore, exosomes can be internalized into recipient cells, in which case they can have two different fates: they can merge into endosomes and undergo transcytosis, or fuse with endosomes maturing into lysosomes, leading to degradation of the exosomal content (Tian *et al.*, 2013).

Exosomes can be found in virtually all body fluids, including saliva, tears, blood, and breast milk (Lasser *et al.*, 2011), and have been shown to be involved in a number of different processes, such as inflammation, diabetes, cardiovascular disease, endothelial dysfunction, coagulopathies, polycystic ovary syndrome, immune modulation and cancer (Lawson *et al.*, 2016; Kalluri, 2016; Slomka *et al.*, 2018).

There is evidence that cancer cells release higher amounts of EVs compared to non-malignant cells as a result of both cell-intrinsic and environmental signals (Bebelman *et al.*, 2018). The former comprises activation of oncogenic signalling pathways, such as EGFRvIII, H-RAS^{V12}, and SRC, which can increase EV production in cancer cells (Al-Nedawi *et al.*, 2008; Lee *et al.*, 2014; Takasugi *et al.*, 2017; Imjeti *et al.*, 2017), and deregulation of the membrane fusion machinery (Wei *et al.*, 2017). The latter mainly involves hypoxia (King, Michael & Gleadle, 2012; Wang *et al.*, 2014a; Li *et al.*, 2016). This evidence is further supported by the observation that blood of cancer patients is twice as rich in exosomes as the blood of healthy individuals, highlighting the potential of exosomes to be used as therapeutic targets or as liquid biopsies to support cancer diagnosis and/or prognosis (Kalluri, 2016).

As a result of activated oncogenic signalling and altered microenvironmental conditions, cancer cells release vesicles that differ in protein and RNA content

from EVs of normal cells (Cha *et al.*, 2015; Ramteke *et al.*, 2015; Griffiths *et al.*, 2017).

Deregulation of cancer EV release and cargo critically impacts the crosstalk between tumour and stroma, both in the tumour microenvironment and at distant metastatic sites (Fig. 1.11). Cho and colleagues observed that tumour-derived exosomes were able to convert mesenchymal stem cells within the stroma of the tumour tissue into cancer-associated myofibroblasts (Cho *et al.*, 2012).

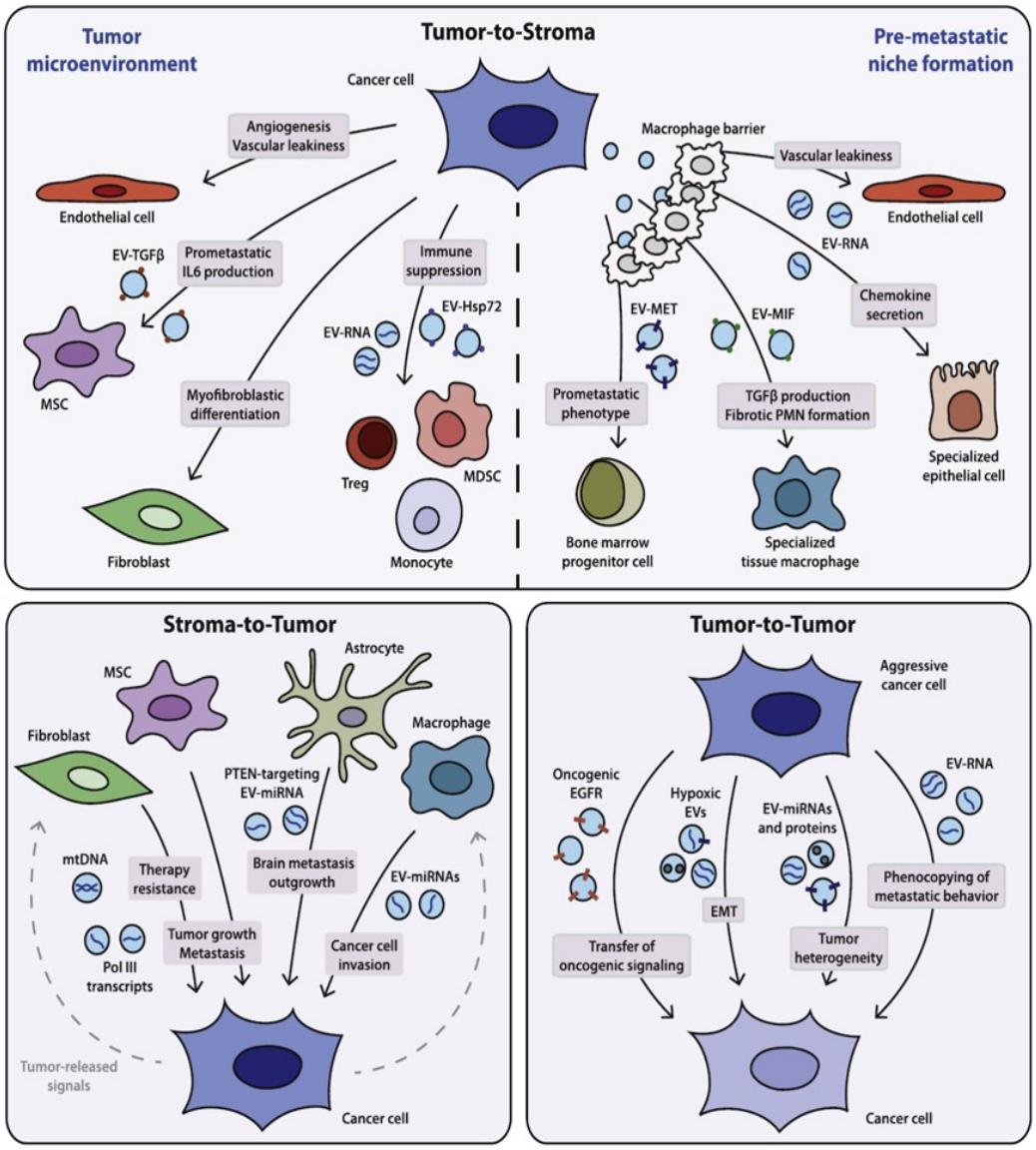


Figure 1.11 Function of EVs in tumour-to-stroma, stroma-to-tumour and tumour-to-tumour communication, fuelling cancer progression. EVs released by cancer cells shape the tumour microenvironment by educating different types of stromal cells to a pro-angiogenic, pro-metastatic and immune suppressive phenotype. Stromal-cell-derived EVs can strongly influence cancer progression by promoting cancer growth and invasive behaviour, as well as outgrowth of cancer cells at the metastatic site and development of therapy resistance. Furthermore, cancer EVs can also transfer malignant traits between

different tumour cell subpopulations, thus propagating tumour heterogeneity. Figure from (Bebelman *et al.*, 2018).

1.9.1 Role of exosomal miRNAs in cancer

miRNAs represent the vast majority of all small RNAs incorporated into exosomes (Goldie *et al.*, 2014).

As has been previously described, miRNA incorporation into exosomes is not a random process, but relies on sorting mechanisms present in the parent cells that guide specific intracellular miRNAs to enter exosomes. In fact, analysis of miRNA expression levels in a variety of cell lines and their derived exosomes led to the discovery that a subset of miRNAs (*e.g.* miR-150, -142-3p, -320, and -451) preferentially enter exosomes (Guduric-Fuchs *et al.*, 2012).

The function of exosomal miRNAs can generally be classified into two types. They can either lead to downregulation of the target genes in the recipient cells, or they can act as ligands that bind to toll-like receptors (TLRs) and activate immune cells, as shown for exosomal miR-21 and miR-29a (Fabbri *et al.*, 2012). However, this latter function has only been recently described, and warrants more investigations in order to fully understand it.

Since the amount and composition of exosomal miRNAs differ between patients with disease and healthy individuals, they show the potential to be used as non-invasive biomarkers to indicate disease state (Zhang *et al.*, 2015). Several studies have profiled miRNAs in different samples and observed that they can be used to aid clinical diagnosis. For instance, levels of exosomal miR-21 were

elevated in serum samples derived from glioblastoma patients compared to healthy individuals (Skog *et al.*, 2008). It was further shown that the levels of exosomal let-7f and/or miR-30e-3p in non-small cell lung cancer (NSCLC) patients can distinguish patients with resectable tumours from those with non-resectable tumours (Silva *et al.*, 2011); similarly, a signature of 8 exosomal miRNAs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214) has been shown to discriminate between patients with malignant tumours and patients with benign tumours, while being absent in healthy controls (Taylor & Gercel-Taylor, 2008). Moreover, levels of 12 exosomal miRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, and miR-214) were significantly different between lung adenocarcinoma patients and healthy controls (Rabinowits *et al.*, 2009). Finally, exosomal let-7, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a have been shown to have the potential to be used as diagnostic biomarkers for colorectal cancer (Ogata-Kawata *et al.*, 2014). Moreover, other candidates, such as miR-1290 and miR-375 in castration-resistant prostate cancer, have been shown to have a prognostic value, thus better allowing to follow disease progression (Huang *et al.*, 2013).

All these observations suggest the application of exosomes and their cargo as clinical tools to diagnose and monitor disease, but much work remains to be done to achieve this goal (Zhang *et al.*, 2015).

1.10 Project aims

Although the genetic landscape of meningioma has been described, and some molecular markers, such as Cyclin D1, have been suggested, meningioma classification is still based on histopathological observation of tumour biopsies, a method subjected to inter-observer variability, and hampered by the fact that tumour tissue might not always be available for histopathological evaluation.

The aims of this project are focussed on:

1. Elucidating microRNA (miRNA)-associated post-translational mechanisms involved in the regulation of Cyclin D1, which has been suggested as a potential prognostic biomarker for meningioma.
2. Analysing and selecting specific miRNA signatures, by comparing WHO I, II, and III meningioma tissues and matched blood samples, in order to identify new prognostic biomarkers circulating in peripheral blood to support the discovery of early tumour recurrence.
3. Determination of the factors responsible for driving deregulation of miRNA candidates, generating potential therapeutic targets.

In order to achieve these aims, open-access databases will be used to identify potential miRNAs targeting Cyclin D1 and the candidates will be validated through RT-qPCR across 125 meningioma tissue samples of all WHO grades and blood samples from 39 meningioma patients. This will allow us to determine the diagnostic value of the candidates in serum exosomes *via* bioinformatics tools. Three post-mortem normal meninges samples will be used as control tissue.

Findings in *ex vivo* samples will then be translated into our *in vitro* meningioma models (WHO I and II primary meningioma cells - PDMN; a benign and a malignant meningioma cell line), which will help us understand the mechanisms involved in the different regulation of miRNA candidates depending on tumour malignancy. From this data, I hope to identify novel potential therapeutic targets for high-grade meningioma patients.

2 Materials and methods

2.1 Cells and exosome biology

2.1.1 Cells and tissues

Meningioma “MN” specimens were collected after surgical procedures when surplus tissue was available. Blood was collected prior to surgery. Patients were consented to the study and given a unique MOT number. This study was granted full ethical approval by the South West research ethic committee (REC No: 14/SW/0119; IRAS project ID: 153351) and local research development approval (Plymouth Hospitals NHS Trust: R&D No: 14/P/056 and North Bristol NHS Trust: R&D No: 3458).

Anonymised meningioma samples under the “J” series were provided by the BRain Archive and Information Network (BRAIN UK) under ethical approval by the South West Research Ethics Committee (REC No: 14/SC/0098; IRAS project ID: 143874, BRAIN UK Ref: 15/011).

Two frozen normal meninges (post mortem) samples were obtained from Analytical Biological Services Inc. and one human brain cerebral meninges (post mortem) was purchased from Novus Biologicals® (NB820-59183; lot B105014) as control tissues.

Human meningeal cells (HMC) were obtained from Sciencell™. The malignant meningioma cell line KT21-MG1-Luc5D (RRID: CVCL_JK00), (Chow *et al.*, 2015) was kindly donated by Dr. J. Chernoff (Fox Chase Cancer Centre, PA, USA) and relabelled from here as “KT21-MG1”. The benign immortalised Ben-

Men-1 cell line was obtained from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Cat# ACC-599, RRID: CVCL_1959) (Puttmann *et al.*, 2005).

2.1.2 Tumour digestion and cell culture

Tumour samples from human meningioma patients were freshly removed by craniotomy and promptly transferred into a sterile tube containing transportation medium composed of Dulbecco's modified Eagle Media (DMEM, Gibco, Life Technologies, Loughborough, UK), supplemented with 10% FBS (Sigma Aldrich, Gillingham, UK), 500 U/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK), and 2.5 µg/mL amphotericin B (Sigma Aldrich, Gillingham, UK). Upon arrival, tumours were washed twice with sterile phosphate buffered saline (PBS, Gibco, Life Technologies, Loughborough, UK), transferred into a P100 sterile plate containing incubation medium (DMEM, Gibco, Life Technologies, Loughborough, UK), supplemented with 10% FBS (Sigma Aldrich, Gillingham, UK), and 100 U/mLpenicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK), and incubated at 37 °C in a humidified atmosphere (5% CO_2) until digestion. Tumours were minced into small pieces and dissociated in DMEM with 10% FBS, 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK) and 20 U/mL Collagenase III (Worthington Biomedical Corp., Lakewood, NJ) overnight at 37 °C. After incubation, cells were pelleted at 1000 rpm for 5 minutes, re-suspended in complete medium, and seeded in appropriate tissue culture flasks. WHO I meningioma primary cells, as well as KT21-MG1 and Ben-Men-1 cell lines,

were routinely cultured in DMEM (Gibco, Life Technologies, Loughborough, UK) supplemented with 10% FBS (Sigma Aldrich, Gillingham, UK), 1% D-(+)-glucose (Sigma Aldrich, Gillingham, UK), 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK) and 2 mM GlutaMAX™-I (Gibco, Life Technologies, Loughborough, UK) at 37 °C, 5% CO_2, maintaining cell confluency above 60%.

WHO II meningioma primary cells were grown in Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (Ham) (DMEM/F-12 (1:1)(1X) + GlutaMAX™-I; Gibco, Life Technologies, Loughborough, UK) supplemented with 20% FBS (Sigma Aldrich, Gillingham, UK), 1% D-(+)-glucose (Sigma Aldrich, Gillingham, UK) and 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK) at 37 °C, 5% CO_2, again maintaining cell confluency above 60%.

Human meningeal cells (HMC) were routinely grown in the recommended HMC medium (Sciencell™, Buckingham, UK) at 37 °C, 5% CO_2.

Schwann cells were grown in poly-L-lysine (PLL, Sigma Aldrich, Gillingham, UK) and laminin (LA, ThermoFisher Scientific, Loughborough, UK) coated 6-well plates in growth factor medium (GFM) composed of DMEM (Gibco, Life Technologies, Loughborough, UK) supplemented with 10% FBS (Sigma Aldrich, Gillingham, UK), 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK), 0.5 µM forskolin (Tocris Bioscience, BioTechne Ltd., Abingdon, UK), 2.5 µg/mL amphotericin (Sigma Aldrich, Gillingham, UK), 2.5 µg/mL insulin (ThermoFisher Scientific, Loughborough, UK), 10 nM β1-heregulin (Rahmatullah *et al.*, 1998) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX-BioTechne, Abingdon, UK).

To freeze cells, all primary cells and cell lines were resuspended in freezing media composed of 50% complete media, 45% FBS, 5% DMSO (Sigma Aldrich, Gillingham, UK) and transferred into cryovials. These were stored at -80 °C overnight in a cryobox (ThermoFisher Scientific, Loughborough, UK) and then transferred into liquid nitrogen for long-term storage.

2.1.2.1 Recovery of cryopreserved cells

Cell samples were rapidly thawed in a 37 °C water bath until complete dissolution of ice. The content of the cryovial was then transferred into a sterile tube and pre-warmed media was added in a drop-wise fashion. Cells were then transferred to either a T25 or T75 flask for amplification. The following day media was changed in order to remove residual DMSO.

2.1.2.2 Cell counting

After collection, cells were pelleted then resuspended in 1 mL of fresh media. Following careful pipetting, 10 µL of the cell suspension were transferred to a haemocytometer counting chamber. 4 sets of 16 squares were counted under a phase contrast microscope with a 10X magnification. Cell concentration (cells/mL) was determined by multiplying the average of the 4 sets by 10^4.

2.1.3 Exosome harvesting

2.1.3.1 From cell culture medium

Exosomes isolation from cell culture medium was performed following the manufacturer's instructions. Briefly, cells were grown in DMEM containing glucose (4.5 g/L), 10% Exo-FBS™ Exosome-depleted FBS (System Bioscience, Cambridge, UK), 100 U/mL penicillin/streptomycin and 1% GlutaMAX™-I (Gibco, Life Technologies, Loughborough, UK) for 72 hours. Media was collected and centrifuged at 2000 x *g* for 30 minutes to remove cell debris, and the supernatant was aliquoted in fresh tubes. 0.5 volumes of the Total Exosome Isolation (from cell culture media) reagent (Invitrogen, ThermoFisher Scientific, Loughborough, UK) was added to the aliquots and samples were incubated at 4 °C overnight.

After incubation, the samples were centrifuged at 10000 × *g* for 1 hour at 4 °C to allow exosome precipitation. After discarding the supernatant, the pellet was re-suspended either in QIAzol Lysis Reagent (Qiagen, Manchester, UK) to allow RNA extraction, or Exosome Re-Suspension Buffer (Invitrogen, ThermoFisher Scientific, Loughborough, UK) and stored at -20 °C ready for downstream processing and analysis.

2.1.3.2 From blood serum

Whole blood was collected in gold Vacutainer® tubes (Becton Dickinson U.K. Ltd., Swindon, UK) and left undisturbed at room temperature to allow clotting

before performing serum isolation. Blood samples were centrifuged at 2400 × *g* for 10 minutes at 4 °C, and serum was aliquoted into 1.5 mL Eppendorf tubes working in a biohazard safety cabinet.

Exosomes isolation from serum was performed following manufacturer's instructions. Samples were centrifuged at 2000 × *g* for 30 minutes to remove cells and debris before the clarified serum was transferred to a new tube and mixed with 0.2 volumes of the Total Exosome isolation (from serum) reagent (Invitrogen, ThermoFisher Scientific, Loughborough, UK) and incubated at 4 °C for 30 minutes.

After incubation, the samples were centifuged at 10000 × *g* for 10 minutes at room temperature to allow exosome precipitation. After discarding the supernatant, the exosome pellet was quickly washed with 1X PBS and then re-suspended in either QIAzol Lysis Reagent (Qiagen, Manchester, UK) to allow RNA extraction, or Exosome Re-Suspension Buffer (Invitrogen, ThermoFisher Scientific, Loughborough, UK) and stored at -20 °C ready for downstream processing and analysis.

2.2 Target identification and validation

2.2.1 Lentivirus-mediated transduction

Cells were plated in 6 well plates until 75% confluent. Media was then changed to a complete medium with 8 µg/mL protamine sulphate (Sigma Aldrich, Gillingham, UK) and lentiviral particles (LentimiRa-GFP-hsa-miR-195-5p cat. No. mh15245 and Lenti-III-mir-GFP control virus cat. No. m002, Applied

Biological Materials Inc., Richmond, BC, Canada; GATA4 shRNA (h) Lentiviral Particles cat. No. sc-35455-V and Control shRNA Lentiviral Particles cat. No. sc-108080, Santa Cruz Biotechnology Inc., Heidelberg, Germany; GATA-4 pLenti-GIII-CMV-GFP-2A-Puro cat. No LVP166711 and control pLenti-CMV-GFP-2A-Puro-Blank cat. No LVP690, Applied Biological Materials Inc., Richmond, BC, Canada) were added in the appropriate volume to reach an MOI of 2. The MOI was calculated using the following formula:

$$\mathrm{MOI} = \frac{Virus\ titre\ \left(\frac{IU}{mL}\right) x\ Virus\ volume\ (mL)}{Total\ cell\ number}$$

Cells were incubated with lentiviral particles for 48 hours, after which they underwent antibiotic selection with complete media containing 10 µg/mL puromycin (ThermoFisher Scientific, Loughborough, UK) for KT21-MG1 cell line and 1 µg/mL puromycin for Ben-Men-1. Cell transduction efficiency was established by monitoring GFP expression, where possible.

After 4 days of selection, cells were harvested using 0.05% Trypsin-EDTA (Gibco, Life Technologies, Loughborough, UK) and plated in white 96-well plates. The day following plating, a viability assay (Cell Titer-Glo® Luminescent Cell Viability Assay kit, Promega, Southampton, UK) was performed in order to determine the effect of the infection on cell viability. Cells were either lysed for protein extraction or resuspended in QIAzol to allow RNA extraction.

2.2.2 *In vitro* pharmacology

PD0332991 isothionate (Palbociclib) was obtained from Sigma Aldrich, Gillingham, UK and resuspended in water. KT21-MG1 cells were plated in 96-well plates and the day following seeding they were treated with Palbociclib 1.25, 2.5, 5 or 10 μM (or vehicle control) for 24 hours (Finn *et al.*, 2009) before monitoring cell viability using a Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega, Southampton, UK).

2-(1,3-benzodioxol-5-ylmethyl)butanedioic acid (Angene International Limited, London, UK) was resuspended in DMSO (Sigma Aldrich, Gillingham, UK). Cells were plated in 96-well plates and treated with a concentration range of 12.5, 25, 50 or 100 μM (plus vehicle control, 0.1% DMSO) (El-Hachem & Nemer, 2011) for 24, 48 and 72 hours to monitor drug effect and choose the best concentrations and time point for combination treatments using a Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega, Southampton, UK).

For combination treatments, cells were pre-treated with 50 or 100 μM of 2-(1,3-benzodioxol-5-ylmethyl)butanedioic acid for 24 hours before media was changed and 1.25, 2.5, 5 or 10 μM Palbociclib were added to the wells for an additional 24 hours, after which cells were either lysed in radioimmunoprecipitation assay (RIPA) buffer or in Qiazol (Qiagen, Manchester, UK) and cell viability was established with a Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega, Southampton, UK). Investigation of possible synergistic, additive or antagonistic effect was performed using the CompuSyn software (Chou, 2010).

2.2.2.1 Cell viability assay

ATP levels were measured as an indicator of viability using the Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega, Southampton, UK, G7570) according to the manufacturer's directions. Briefly; cells were plated in triplicate into a white, flat bottom 96-well plate at a density of 3500 cells per well. For drug treatments, the following day cells were treated with the desired drug concentrations for the selected duration. At the end of the time point, media was removed and replaced with 100 µL of fresh media, then 100 µL of Cell Titer-Glo® reagent were added per well. Cells were then placed on a plate shaker for 2 minutes at 450 rpm. Following a further incubation for 10 minutes at room temperature to allow signal stabilization, luminescence was measured using the BMG Labtech FLUOstar® Omega plate reader (BMG Labtech, Aylesbury, UK).

2.3 Protein analysis

2.3.1 Western blotting

Protein extraction, quantification and Western blotting were performed as previously described (Dunn *et al.*, 2019).

2.3.1.1 Protein extraction and quantification

Cultured cells were washed three times with ice cold PBS before being lysed in RIPA lysis buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium

dodecyl sulphate - SDS, 1% NP40, 50 nM Tris-HCl; pH 8) containing complete protease inhibitor (cOmplete™, EDTA free Protease Inhibitor Cocktail, Sigma-Aldrich®; #11873580001; 1:20) and phosphatase inhibitors cocktail (Santa Cruz Biotechnology, Inc., Heidelberg, Germany; Sc-45,045; sc-45,065; 1:100). Following mechanical scraping, lysates were collected and frozen at −80 °C for at least 24 hours before being thawed on ice and centrifuged at 15,000 × *g* for 15 minutes at 4 °C. The supernatant was transferred to fresh tubes and protein concentration was determined using the colorimetric Pierce bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific, Loughborough, UK). A standard curve was calculated using serial dilutions of Bovine Serum Albumin (BSA – 2mg/mL) provided with the kit (stock concentrations used were 0, 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/mL; BSA was diluted in lysis buffer).

Whole protein lysates were diluted 1:4 in PBS and 12 µL of the standard and samples were pipetted in duplicates into clear 96-well plates; 200 µL of the reagent (A+B, 1:50) were added to each well. The plate was then incubated at 37 °C for 20 minutes before measuring absorbance at 562 nm on a microplate reader (BMG Labtech plate FLUOstar® Omega plate reader, BMG Labtech, Aylesbury, UK).

The mean absorbance value of the blank was subtracted from the mean of all the standards and unknown samples; the average blank corrected values were then used to plot the standard curve and obtain the linear equation which was used to determine the protein concentration of the unknown samples using Microsoft Excel.

2.3.1.2 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated based on their size by a gel electrophoresis using a discontinuous polyacrylamide gel system. A 4% stacking gel (Table 2.1) was layered on top of an 8% or 10% resolving gel (Table 2.2), depending on the size of the protein of interest.

40 µg of protein per sample were mixed with 4X loading buffer (250 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 4% β-mercaptoethanol, 0.4% bromophenol blue) and the samples were heated at 95°C for 5 minutes. The samples were then cooled on ice, loaded onto the acrylamide gels and run at 40 mA in 1X Running Buffer (Table 2.3). 10 µL of Precision Plus Prestained Dual Color Protein ladder (BioRad, Watford, UK) were loaded as a standard size of protein.

10 mL Stacking Gel	**4%**
Protogel 40% (BioRad, Watford, UK)	1 mL
0.5 M Tris-HCl, 0.4% SDS, pH 6.8	2.5 mL
milliQ water	6.38 mL
10% APS (Sigma Aldrich, Gillingham, UK)	100 µL
TEMED (ThermoFisher Scientific, Loughborough, UK)	10 µL

Table 2.1 Stacking gel recipe.

20 mL Resolving gel	8% (25-200 kDa)	10% (15-100 kDa)
ProtoGel 40% (BioRad, Watford, UK)	4 mL	5 mL
1.5 M Tris-HCl pH 8.8	5 mL	5 mL
10% SDS (Fisher Scientific, Loughborough, UK)	200 µL	200 µL
milliQ water	10.58 mL	9.58 mL
10% APS (Sigma Aldrich, Gillingham, UK)	200 µL	200 µL
TEMED (ThermoFisher Scientific, Loughborough, UK)	20 µL	20 µL

Table 2.2 Resolving gel recipe.

	Running Buffer 10X	Transfer Buffer 10X
Glycine (ChemCruz, Santa Cruz Biotechnology, Inc, Heidelberg, Germany)	144 g	144 g
Trizma Base (Sigma Aldrich, Gillingham, UK)	30.2 g	30.2 g
20% SDS (Fisher Scientific, Loughborough, UK)	50 mL	-
milliQ water	Up to 1 L	Up to 1 L

Table 2.3 Buffer recipes.

2.3.1.3 Protein transfer and immunoassay

Separated proteins were transferred onto a PVDF membrane (Bio-Rad), following activation in methanol, at 400 mA for 70 minutes in 1X Transfer buffer (525 mL milliQ water, 70 mL 10X Transfer Buffer – Table 2.3, 105 mL methanol).

After transfer, membranes were blocked with 5% skimmed milk or 5% BSA (ThermoFisher Scientific, Loughborough, UK) in PBS with 0.05% Tween-20 (PBST) for one hour. Following blocking, membranes were incubated overnight at 4 °C on a shaker with the primary antibodies listed in Table 2.4.

Antibody	**Company**	**Species**	**Catalogue #**	**Dilution**
Cyclin D1 (92G2)	CST, London, UK	Rabbit	2978T	1:1000
Cyclin D2 (D52F9)	CST, London, UK	Rabbit	3741T	1:1000
Cyclin D3 (DCS22)	CST, London, UK	Mouse	2936T	1:1000
Cyclin E1 (HE12)	CST, London, UK	Mouse	4129T	1:1000
CDK2 (78B2)	CST, London, UK	Rabbit	2546T	1:1000
CDK4 (D9G3E)	CST, London, UK	Rabbit	12790T	1:1000
CDK6 (D4S8S)	CST, London, UK	Rabbit	13331T	1:1000
GATA-4 (D3A3M)	CST, London, UK	Rabbit	36966S	1:500
GATA-5	Abcam, Cambridge, UK	Rabbit	ab48820	1:500
GATA-6 (D61E4) XP®	CST, London, UK	Rabbit	5851T	1:500

FOG-2	Abcam, Cambridge, UK	Rabbit	ab123879	1:500
Bcl-2 (124)	CST, London, UK	Mouse	15071T	1:1000
RB [EPR17512]	Abcam, Cambridge, UK	Rabbit	ab181616	1:500
pRB (Ser807/811) (D20B12) XP®	CST, London, UK	Rabbit	8516S	1:500
CD9 (D8O1A)	CST, London, UK	Rabbit	13174S	1:500
CD63 (MX-49.129.5)	Santa Cruz Biotechnology Inc., Heidelberg, Germany	Mouse	sc-5275	1:500
GM130 (35/GM130)	Becton Dickinson U.K. Ltd., Swindon, UK	Mouse	610823	1:500
Calnexin (H-70)	Santa Cruz Biotechnology Inc., Heidelberg, Germany	Rabbit	sc-11397	1:1000
GAPDH (6C5)	Millipore, Watford, UK	Mouse	MAB374	1:50000

Table 2.4 Primary antibodies.

The following day, membranes were washed three times in PBS-T before incubation with the appropriate HRP-conjugated secondary antibody (Table 2.5) in 5% skimmed milk or 5% BSA for 1 hour before further washing.

Antibody	**Type**	**Company**	**Catalogue #**	**Dilution**
Goat anti-Rabbit	HRP conjugated	BioRad, Watford, UK	170-6516	1:1000
Goat anti-Mouse	HRP conjugated	BioRad, Watford, UK	172-1019	1:1000 - 1:5000

Table 2.5 Secondary antibodies.

Proteins were detected using the Pierce ECL Western blotting substrate or Pierce ECL Plus Western blotting substrate (ThermoFisher Scientific, Loughborough, UK) depending on signal strength, using ECL hyperfilm (GE Healthcare, Chalfont Saint Giles, UK) and a Xenograph compact X4 X-ray film processor (ThermoFisher Scientific, Loughborough, UK). Films were scanned at a resolution of 600 dpi using an HP Scanjet 2400 and band densities were quantified and normalised to GAPDH using the ImageJ (Fiji) software (Schneider, Rasband & Eliceiri, 2012).

Membrane stripping was performed by rinsing the blot in water followed by incubation with wash buffer (100 mM glycine pH 2.4, 2 mM EGTA in H_2O) for 5 minutes prior to incubation with stripping buffer (31 mL 1M Tris-HCl pH 6.8, 100 mL 10% SDS, 3505 µL β-mercaptoethanol in 365.5 mL H_2O) pre-heated to 56

°C, for five minutes. After this, membranes were re-blocked and re-probed with primary antibodies.

2.3.2 Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were cut in 4 µm thick sections by the Department of Cellular Pathology at University Hospitals Plymouth, transferred onto histology slides, and baked at 60 °C to adhere. Sections were de-waxed through xylene (ThermoFisher Scientific, Loughborough, UK) and 100% ethanol (VWR, Lutterworth, UK) before being rinsed under tap water for 5 minutes and being blocked in 3% hydrogen peroxide in methanol for a minimum of 30 minutes to quench endogenous peroxidase activity.

Antigen retrieval was performed by microwaving the slides in a solution of Tris/EDTA for 30 minutes. After equilibration in TBS/Tween wash buffer for 5 minutes, slides were incubated with the primary antibody overnight (GATA-4 25310, LOT A1218, Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:100 in TBS/Tween) at room temperature in a moist, sealed chamber. Proteins were visualised with the Novolink Polymer detection system for Leica (Leica Biosystems, Newcastle, UK, RE 7140-K) according to the manufacturer's instructions. 3'3'-diaminobenzidine (DAB, Sigma Adrich, Gillingham, UK) was used for visualisation and followed by incubation for 5 minutes in a copper sulphate DAB enhancer solution.

Nuclei were then lightly counterstained in Mayer's hematoxylin for 2 minutes before slides were dehydrated through 100% ethanol and xylene prior to mounting and cover-slipping using Pertex (Sigma Aldrich, Gillingham, UK,

Gillingham, UK). As a negative control, sections were incubated as described above with omission of the primary antibody.

Semi-quantitative assessment of the staining intensity was carried out blinded by consultant neuropathologist Dr David Hilton (Department of Cellular and Anatomical Pathology, University Hospitals Plymouth), and assigned as follows: 0 if negative, 1 if low immunoreactivity was observed, 2 if the signal was moderate and 3 in case of a strong immunoreactivity (Dunn *et al.*, 2019).

2.4 Polymerase chain reaction (PCR)

2.4.1 miRNA candidates identification

The following open-access databases were used to find potential miRNA candidates: TargetScanHuman7.2, http://www.targetscan.org/vert_72/; DIANATOOLS microT-CDS, http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index; mirDIP, http://ophid.utoronto.ca/mirDIP/; MicroRNA Target prediction tool - miRTAr, http://mirtar.mbc.nctu.edu.tw/human/; UTRdb tool, http://utrdb.ba.itb.cnr.it/.

2.4.2 RNA extraction

Total RNA was extracted from exosomes, cell lines, and primary meningioma cells and tissues using the phenol-chloroform method, following manufacturer's directions. Cells were harvested using 0.05% Trypsin-EDTA (ThermoFisher

Scientific, Loughborough, UK) and washed once in 1X PBS. The pellet was resuspended in 500 µL QIAzol Lysis Reagent (Qiagen, Manchester, UK) and frozen at -20 °C overnight. The following day, 100 µL chloroform were added to the samples and the tubes were shaken for 15 seconds. Samples were then centrifuged at 12000 × *g* for 15 minutes at 4 °C. The upper clear phase was transferred to a new tube, mixed with 250 µL isopropanol and centrifuged again at 12000 × *g* at 4 °C for 10 minutes. The supernatant was discarded and 500 µL of 75% ethanol were added to the RNA pellet. The tubes were spun at 7500 × *g* for 5 minutes at 4 °C, then an appropriate volume of nuclease free water (not DEPC-treated, Invitrogen, ThermoFisher Scientific, Loughborough, UK) (20-100 µL) was added to the RNA pellet, and samples were incubated for 10 minutes at 56 °C to allow rehydration. RNA samples were then stored at -80 °C. Quantification was carried out using a NANODROP 2000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK).

2.4.3 Real Time PCR: microRNA expression analysis

Two-step RT-qPCR (qPCR) was performed using TaqMan® microRNA assays (RNU6B ID 001093, hsa-miR-195* ID 002107, hsa-miR-15* ID 002419, hsa-miR-15b* ID 002173, hsa-miR-16-1* ID 002420, and hsa-miR-497* ID 002368; Applied Biosystems, ThermoFisher Scientific, Loughborough, UK) according to manufacturer's directions.

RT-PCR was performed starting from 1 µg of total RNA using the TaqMan® MicroRNA Reverse Transcription Kit (10X RT Buffer, MultiScribe™ RT enzyme

50U/μL, RNase inhibitor 20U/μL, dNTP mix with dTTp 100μM total, Applied Biosystems, ThermoFisher Scientific, Loughborough, UK).

The program used was as follows:

1. 16 °C, 30 minutes
2. 42 °C, 30 minutes
3. 85 °C, 5 minutes
4. 4 °C, ∞.

After RT-PCR, the samples were quickly centrifuged at 4 °C, and stored at -20 °C until qPCR was performed, using the LightCycler® 480 II System (Roche Products Limited, Welwyn Garden City).

For qPCR, samples were diluted with nuclease free water and plated in a clear 96-well plate (Roche Products Limited, Welwyn Garden City) with the qPCR mix (TaqMan® 20X probes, TaqMan® Fast Advanced Master Mix, Applied Biosystems, ThermoFisher Scientific, Loughborough, UK).

qPCR was performed in triplicate for each gene with the following program:

1. Hotstart: 95 °C, 2 minutes
2. qPCR amplification (45 cycles):
    1. 95 °C, 15 seconds
    2. 60 °C, 1 minute
    3. Cooling: 40 °C, 30 seconds.

All miRNA expression levels were normalized to the expression of the small nuclear U6 RNA (RNU6B); RNA extracted from the HMC cell line was used as calibrator. The quantitation method used was $2^{-(\Delta\Delta Ct)}$ (Livak & Schmittgen, 2001).

2.4.4 Real Time qPCR: gene expression analysis

RT-PCR was performed starting from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (10X RT Buffer, 25X dNTP mix, 10X Random primers, MultiScribe® Reverse Transcriptase 50U/µL, RNase inhibitor 20U/µL, Applied Biosystems, ThermoFisher Scientific, Loughborough, UK) according to manufacturer's instructions.

The High-Capacity RT-PCR program was as follows:

1. 25 °C, 10 minutes
2. 37 °C, 120 minutes
3. 85 °C, 5 minutes
4. 4 °C, ∞.

After RT-PCR, the samples were quickly centrifuged and stored at -20 °C.

qPCR with TaqMan® probes (CCND1 ID Hs00765553_m1, CCND2 ID Hs00153380_m1, CCND3 ID Hs00236949_m1, CCNE1 ID Hs01026536_m1, GATA-4 ID Hs00171403_m1, Bcl-2 ID Hs01048932_g1, and GAPDH ID Hs02786624_g1; Applied Biosystems, ThermoFisher Scientific, Loughborough, UK) was performed in triplicate for each gene. To do so, samples were diluted with nuclease free water and plated in a clear 96-well plate (Roche Products Limited, Welwyn Garden City), with the qPCR mix, which was composed of TaqMan® 20X probes and TaqMan® Fast Advanced Master Mix (Applied Biosystems, ThermoFisher Scientific, Loughborough, UK).

The program used was:

1. Hotstart: 95 °C, 2 minutes
2. qPCR amplification (45 cycles):
    1. 95 °C, 15 seconds
    2. 60 °C, 1 minute
    3. Cooling: 40 °C, 30 seconds.

All gene expression levels were normalized to the expression of the housekeeping gene GAPDH; RNA extracted from the HMC cell line was used as a calibrator. The quantitation method used was $2^{-(\Delta\Delta Ct)}$ (Livak & Schmittgen, 2001).

2.4.5 ROC curve analysis

ROC curves were created by comparing RT-qPCR results of grade I and II-III meningioma samples using the data analysis software GraphPad Prism. The area under the curve was used to quantify the overall ability of the test to discriminate between the two groups (Carter *et al.*, 2016).

An AUC of 1 represents a perfect test, of 0.9-0.99 an excellent test, 0.8-0.89 a good test, 0.7-0.79 a fair test, 0.51-0.69 a poor test, while 0.5 a test of no value (Hanley & McNeil, 1982; Cook, 2007).

The software plots sensitivity on the y-axis, which is defined as the proportions of positives which are identified correctly as such, *i.e.* true-positive rate (= true positives/[true positives + false negatives]), whereas 1-specificity on the x-axis

which represents the false positive rates *i.e.* the proportion of incorrect positive results that are in fact negative (Carter *et al.*, 2016).

The blue diagonal line in graphs (from coordinates x = 0, y = 0 to x = 1, y = 1) is known as line of identity or random chance line; the closer an ROC curve is to the line of identity, the less accurate the test; values plotted above and to the left of this line represent correctly predictive results, whereas values laying below and to the right represent incorrectly predictive results (Carter *et al.*, 2016).

2.5 Statistical analysis

GraphPad Prism data analysis software was used throughout the project to calculate the statistical significance in each experiment. Experiments were performed at least in triplicate unless otherwise mentioned:

- Western blot: biological triplicate for cell lines, n biological replicate for primary cells and tissues.
- RT-qPCR: technical and biological triplicate for cell lines, technical triplicate and n biological repeats for cells, tissues and exosomes.
- Functional studies, drug testing: biological triplicate.

Statistical analysis was performed via unpaired or paired t-Test in experiments with two different groups. In experiments with three or more different groups data was analysed with one-way ANOVA and Tukey's Multiple Comparison as a post-test.

Significance levels are denoted throughout the thesis as follows: NS = not significant ($p>0.05$), * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$. Data are expressed as mean ± standard error of the mean (SEM).

3 Results

3.1 Identification of a miRNA cluster downregulated in malignant meningioma

Introduction

Despite the genetic landscape of meningioma having been described, meningioma classification is still based on histopathological observation of tumour biopsies, which is subjected to inter-observer variability and has been questioned due to the little prognostic effect of the histological criteria (Rogers *et al.*, 2016; Vaubel *et al.*, 2016; Baumgarten *et al.*, 2016).

Cyclin D1 has been suggested as a molecular biomarker for meningioma (Alama *et al.*, 2007; Milenkovic *et al.*, 2008) and a growing body of evidence showed that microRNAs can have diagnostic and/or prognostic significance in meningioma (Kliese *et al.*, 2013; Zhi *et al.*, 2013; Ludwig *et al.*, 2015; Wang *et al.*, 2015; Zhi *et al.*, 2016; El-Gewely *et al.*, 2016).

In this section, I assessed the expression levels of Cyclin D1 in our meningioma tissue samples, primary cells and cell lines in order to understand whether it is altered, as suggested by previous literature (Alama *et al.*, 2007; Milenkovic *et al.*, 2008). Subsequently, using open-access databases, I searched for miRNAs which are involved in the translational regulation of Cyclin D1 in meningioma, and the candidates were validated through RT-qPCR in 125 meningioma tissue samples of all grades, primary meningioma cells and cell lines. The diagnostic

value of the candidates has been assessed via receiver operating characteristic (ROC) curve analysis.

3.1.2 Cyclin D1 is overexpressed in malignant meningioma

I assessed the protein levels of Cyclin D1 (and also D2, D3 and E1, which share common translational regulators with Cyclin D1, see below) in *ex-vivo* samples and in our *in vitro* model of meningioma (patient-derived meningioma - PDMN - cells, Ben-Men-1, and KT21-MG1 cells) *via* Western blotting, as described in section 2.3.1. Two normal meningeal tissue lysates and normal human meningeal cells (HMC) have been used as a control.

Western blot analysis revealed an increased trend of Cyclin D1, D2, D3 and E1 levels in WHO I, II and III meningioma tissue samples compared to normal meninges (Fig. 3.1A). Some of the tissue samples considered in the quantification have been analysed by Dr Daniele Baiz, as they were not available anymore for me to assay.

Interestingly, I observed that only Cyclin D1 is significantly overexpressed in PDMN cells compared to control (HMC cells, 1.5 fold changes), while there is an increased trend in Cyclin D2 expression in PDMN compared to HMC. However, Cyclin D3 and E1 levels remained unchanged (Fig.3.1B).

In addition, I assessed Cyclin D1, D2, D3 and E1 in the WHO I meningioma cell line Ben-Men-1 compared to the malignant meningioma cell line KT21-MG1. Whilst Cyclin D1 levels remained unchanged, Cyclin D2 was not detected in the benign cell line and the expression levels of Cyclin D3 and E1 were 40% and

26% higher in Ben-Men-1 compared to HMC, respectively. Conversely, all Cyclins were significantly upregulated in the malignant cell line when compared to both HMC and Ben-Men-1 (Fig. 3.1C).

Differences observed in Cyclins expression in the controls used, normal meninges and HMC, are probably due to their different nature (Fig. 3.1A and B); in fact, the normal meningeal tissues used are post mortem samples of human origin, whereas HMC is a cell line of foetal origin, cultured in a very rich media to allow proliferation.

I also carried out a gene expression analysis on Ben-Men-1 and KT21-MG1 cells, as described in section 2.4.4, and observed that *Cyclin D1, D2, and E1* are significantly overexpressed in the malignant cell line at the transcriptional level as well as the protein level, whereas *Cyclin D3* is practically unchanged between the two samples (Fig. 3.1D).

Taken together, these results suggested that Cyclin D1 is overexpressed in the meningioma samples analysed in this study, when compared to controls and as published previously (Alama *et al.*, 2007; Milenkovic *et al.*, 2008).

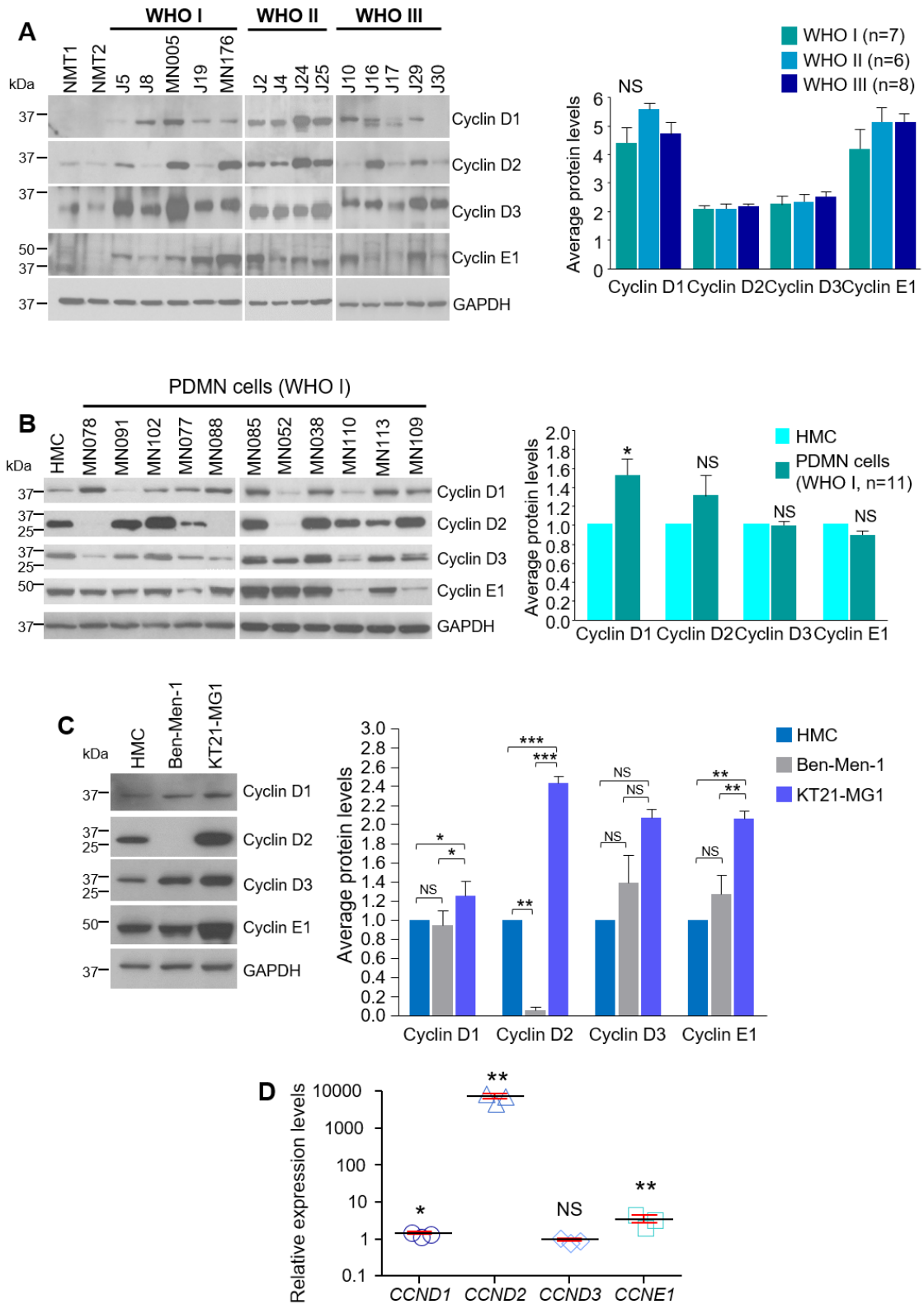


Figure 3.1 Investigation of Cyclin expression levels in meningioma by Western blot and gene expression analysis. (A) Immunoreactivity and relative protein quantification of Cyclin D1, D2, D3, and E1 in meningioma

tumours (n = 21, WHO I = 7, WHO II = 6, WHO III = 8). All samples have been normalized against the average protein expression in two normal meninges lysates and presented in a Log_2 scale. (B) Immunoreactivity and relative protein quantification of Cyclin D1, D2, D3, and E1 in WHO I PDMN cells (n = 11) and HMC. Only Cyclin D1 displayed a significant increase in PDMN compared to HMC. All data have been normalized against the average expression in HMC and presented in a linear scale. An HMC lane was present in both panels. (C) Representative Western blot and relative protein quantification of Cyclin D1, D2, D3, and E1 in the WHO I meningioma cell line Ben-Men-1 and the malignant meningioma cell line KT21-MG1. One out of three Western blots is shown; protein levels were normalized against the loading control GAPDH and the relative quantification compared to HMC is reported. (D) Relative gene expression analysis conducted by qPCR of *Cyclin D1, D2, D3, and E1* in Ben-Men-1 and KT21-MG1. Data shown have been normalized against the average expression levels in Ben-Men-1 and presented in a Log_{10} scale as mean ± SEM. Every dot represents a different passage of the same cell line. (NS = not significant; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$).

Since I observed Cyclin D1, D2, D3, and E1 upregulation in KT21-MG1 compared to both Ben-Men-1 and HMC, I decided to assess the expression levels of the Cyclin-dependent kinases 4 and 6 (CDK4/6), which interact with proteins belonging to the Cyclin D family to exert their functional roles, *via* Western blotting (see 2.3.1).

Interestingly I observed that CDK4 was overexpressed in KT21-MG1 cells compared to Ben-Men-1 and HMC, while CDK6 expression did not change (Fig. 3.2).

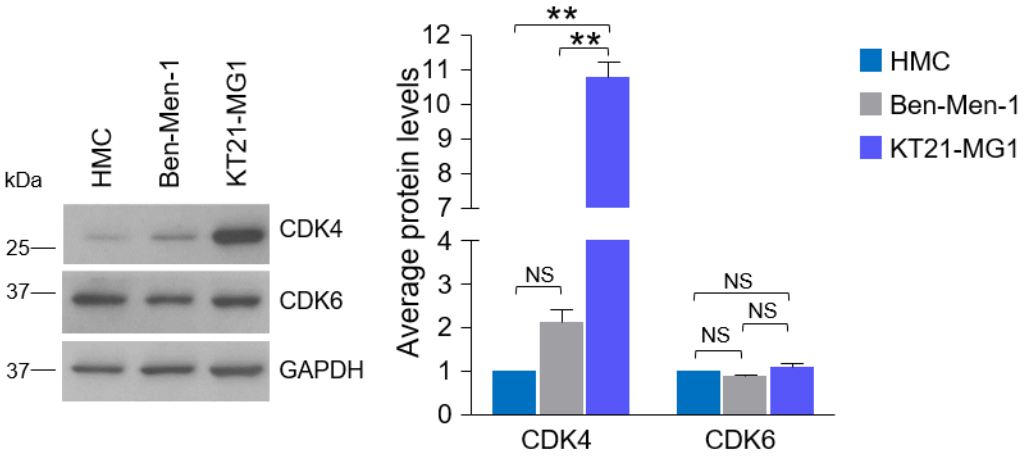


Figure 3.2 CDK4 is overexpressed in KT21-MG1 cells. Representative Western blot and relative protein quantification showing overexpression of CDK4 in KT21-MG1 cells compared to both Ben-Men-1 (5.2 folds) and HMC. (NS = not significant, ** = $p<0.01$).

3.1.3 Cyclin D1 is targeted by the miR-15 family

Through the TargetScanHuman7.2 algorithm, with help from my supervisor, Dr Daniele Baiz, I identified the miR-15 family as a suitable candidate to be investigated in meningioma, as it has previously been experimentally validated that members of the miR-15 family are able to bind to and downregulate *Cyclin D1* (and also *D2, D3, and E1*) mRNAs (Furuta *et al.*, 2013; Wei *et al.*, 2016; Yin *et al.*, 2017).

The miR-15 family is composed of six members which can be found throughout the genome in three different clusters: miR-15a~16-1, miR-15b~16-2, and miR-497~195. As shown in Table 3.1, the miR-16-1 and -16-2 share the same sequence and were both profiled in this study as “miR-16-1“ (Aqeilan, Calin & Croce, 2010; Porrello *et al.*, 2011).

Cluster	Location	Name and sequence of mature miRNA
miR-15a~16-1	13q14.3	miR-15a-5p 5’ 14 – uagcagcacauaaugguuugug – 35 3’ miR-16-1-5p 5’ 14 – uagcagcacguaaauauuggcg – 35 3’
miR-15b~16-2	3q26.1	miR-15b-5p 5’ 20 – uagcagcacaucaugguuuaca – 41 3’ miR-16-2-5p 5’ 10 – uagcagcacguaaauauuggcg – 31 3’
miR-497~195	17p13.1	miR-497-5p 5’ 24 – cagcagcacacugugguuugu – 44 3’ miR-195-5p 5’ 15 – uagcagcacagaaauauuggc – 35 3’

Table 3.1 miR-15 family members. Notably all members share the same seed sequence (represented in red), while miR-16-1 and -16-2 share the exact same sequence and will thus be referred to as “miR-16-1-5p” (TargetScanHuman7.2 algorithm; Lewis, Burge & Bartel, 2005).

Table 3.2 shows all potential miR-15 family members binding sites on *CCND1, D2, D3 and E1* mRNAs.

	miRNA	Position in the UTR	seed match	context++ score	context++ score percentile	weighted context++ score	conserved branch length	Pct
	Conserved sites							
CCND1	hsa-miR-16-5p	1961-1967	7mer-1A	-0.19	86	-0.18	4.044	0.71
	hsa-miR-195-5p	1961-1967	7mer-1A	-0.19	86	-0.18	4.044	0.71
	hsa-miR-15a-5p	1961-1967	7mer-1A	-0.17	83	-0.16	4.044	0.71
	hsa-miR-497-5p	1961-1967	7mer-1A	-0.15	82	-0.14	4.044	0.71
	hsa-miR-15b-5p	1961-1967	7mer-1A	-0.16	81	-0.15	4.044	0.71
	hsa-miR-15a-5p	2033-2040	8mer	-0.44	99	-0.42	4.262	0.84
	hsa-miR-16-5p	2033-2040	8mer	-0.43	99	-0.41	4.262	0.84
	hsa-miR-195-5p	2033-2040	8mer	-0.43	99	-0.41	4.262	0.84
	hsa-miR-497-5p	2033-2040	8mer	-0.46	99	-0.44	4.262	0.84
	hsa-miR-15b-5p	2033-2040	8mer	-0.43	99	-0.41	4.262	0.84
CCND2	hsa-miR-497-5p	620-626	7mer-1A	-0.23	91	-0.2	6.165	0.86
	hsa-miR-16-5p	620-626	7mer-1A	-0.18	85	-0.15	6.165	0.86
	hsa-miR-195-5p	620-626	7mer-1A	-0.18	84	-0.15	6.165	0.86
	hsa-miR-15b-5p	620-626	7mer-1A	-0.17	83	-0.14	6.165	0.86
	hsa-miR-15a-5p	620-626	7mer-1A	-0.17	83	-0.14	6.165	0.86
	hsa-miR-16-5p	1693-1699	7mer-1A	-0.17	84	-0.15	7.513	0.86
	hsa-miR-195-5p	1693-1699	7mer-1A	-0.17	83	-0.15	7.513	0.86
	hsa-miR-497-5p	1693-1699	7mer-1A	-0.16	82	-0.13	7.513	0.86
	hsa-miR-15a-5p	1693-1699	7mer-1A	-0.15	80	-0.13	7.513	0.86
	hsa-miR-15b-5p	1693-1699	7mer-1A	-0.13	78	-0.12	7.513	0.86
	hsa-miR-497-5p	1764-1771	8mer	-0.3	96	-0.26	7.513	0.9
	hsa-miR-15b-5p	1764-1771	8mer	-0.25	92	-0.21	7.513	0.9
	hsa-miR-15a-5p	1764-1771	8mer	-0.25	92	-0.21	7.513	0.9
	hsa-miR-195-5p	1764-1771	8mer	-0.23	90	-0.2	7.513	0.9
	hsa-miR-16-5p	1764-1771	8mer	-0.23	90	-0.2	7.513	0.9
CCND3	hsa-miR-497-5p	768-774	7mer-1A	-0.27	94	-0.27	5.837	0.86
	hsa-miR-15a-5p	768-774	7mer-1A	-0.25	92	-0.25	5.837	0.86
	hsa-miR-15b-5p	768-774	7mer-1A	-0.25	92	-0.25	5.837	0.86
	hsa-miR-195-5p	768-774	7mer-1A	-0.19	86	-0.19	5.837	0.86
	hsa-miR-16-5p	768-774	7mer-1A	-0.19	86	-0.19	5.837	0.86
CCNE1	hsa-miR-497-5p	247-254	8mer	-0.42	98	-0.42	3.917	0.81
	hsa-miR-16-5p	247-254	8mer	-0.39	98	-0.39	3.917	0.81
	hsa-miR-15b-5p	247-254	8mer	-0.4	98	-0.4	3.917	0.81
	hsa-miR-15a-5p	247-254	8mer	-0.4	98	-0.4	3.917	0.81
	hsa-miR-195-5p	247-254	8mer	-0.39	98	-0.39	3.917	0.81
	hsa-miR-15a-5p	485-492	8mer	-0.63	99	-0.63	4.501	0.86
	hsa-miR-15b-5p	485-492	8mer	-0.63	99	-0.63	4.501	0.86
	hsa-miR-497-5p	485-492	8mer	-0.65	99	-0.65	4.501	0.86
	hsa-miR-195-5p	485-492	8mer	-0.65	99	-0.65	4.501	0.86
	hsa-miR-16-5p	485-492	8mer	-0.65	99	-0.65	4.501	0.86

Table 3.2 miR-15 family members bind to the 3'UTR regions of Cyclins mRNAs. The 3' UTRs of *CCND1, CCND2, and CCNE1* show the highest affinity with miR-15 family members as they contain 8mer seed matches. *CCND3* 3' UTR only contains 7mer-1A seed matches (exact match to positions 2 - 7 of the mature miRNA followed by an adenine).

3.1.4 Investigation of miR-15 family members levels in meningioma tissues

To investigate the levels of miR-15 family members in meningioma tissues, I performed a gene expression qPCR analysis of mature miRNAs on total RNA extracted from 83 WHO I, 30 WHO II, and 12 WHO III meningioma samples, as described in section 2.4.3. Three healthy meninges were used as a calibrator, *i.e.* the sample that all others were compared to. As an endogenous control, I used the snRNA U6.

Interestingly, I observed that the expression levels of miR-15a, -16-1, and -15b were practically unchanged in WHO I, II, and III meningioma tissue samples analysed (Fig. 3.3A, B, and C, respectively). However there was a significant downregulation of miR-497 and -195 in WHO II (-0.49 and -0.55 Log_{10} fold decrease, respectively) and malignant samples compared to WHO I (-1.40 and -1.04 Log_{10} fold decrease, respectively) (Fig. 3.3D and E, respectively).

I assessed the diagnostic value of miR-497 and -195 through receiver operating characteristic (ROC) curve analysis, by comparing WHO I meningioma tissues to WHO II-III samples grouped together, in order to understand the efficiency of the two candidates in classifying patients affected by benign tumours and those affected by higher-grade tumours (see section 2.4.5). Despite the number of patients profiled (n = 125, WHO I = 83, WHO II = 30, WHO III = 12), both miR-497 and -195 show potential to be good diagnostic biomarkers in meningioma tissues, with an area under the curve (AUC) of 0.7946 and 0.8498, respectively (Fig. 3.3F and G, respectively).

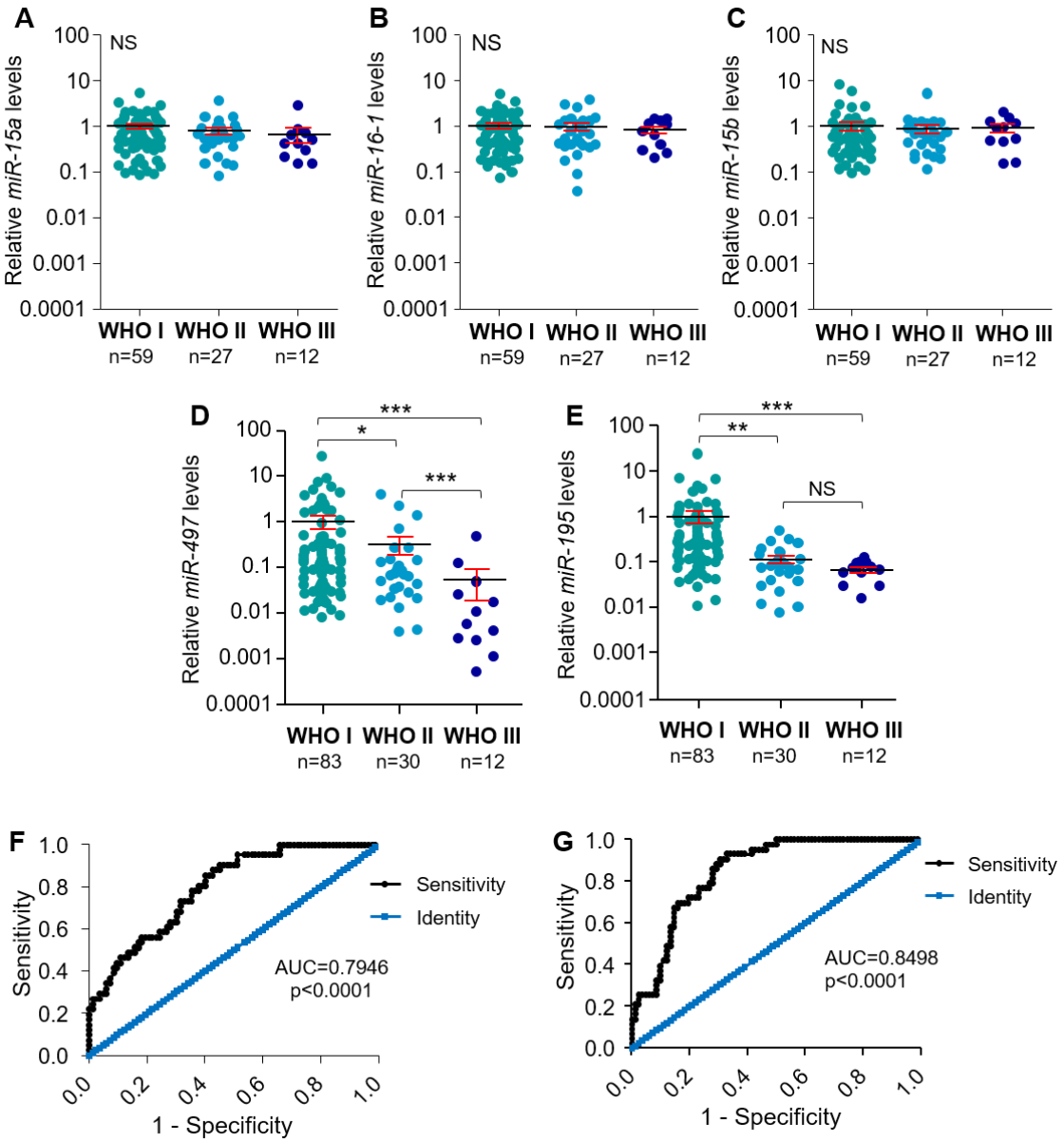


Figure 3.3 Gene expression analysis performed by qPCR of the miR-15 family in meningioma tissues. (A, B, C) Expression levels of miR-15a, -16-1, and -15b in tumours, respectively (n = 98; WHO I = 59, WHO II = 27, WHO III = 12). miRNA levels were normalised to the mean of WHO I meningioma samples, and shown as mean ± SEM in a Log_{10} scale. (D, E) Expression levels of miR-497 and -195 in meningioma tumour samples, respectively (n = 125; WHO I = 83, WHO II = 30, WHO III = 12). miRNA levels were normalised against the mean of WHO I meningioma samples, and shown as mean ± SEM

in a Log_{10} scale. (NS = not significant; * = p<0.05; ** = p<0.01; *** = p<0.005). (F, G) Receiver operating characteristic (ROC) curves showing the diagnostic value of miR-497 and -195, respectively (n = 125). AUC values shown in figure, 95% confidence interval = 0.7159 to 0.8733 and 0.7847 to 0.9150, respectively. ROC curves were calculated by comparing the expression levels of miR-497 and -195 in WHO II and III tissues grouped together with those in grade I samples.

As both miR-497 and -195 singularly showed a good diagnostic value in distinguishing between benign (WHO I) and higher-grade meningiomas (WHO II-III), I further analysed the combined diagnostic value of these miRNAs. When miR-497 and -195 are considered as a 2-miRNA signature, ROC curve analysis revealed a good tissue diagnostic value, with an area under the curve of 0.8102 (Fig. 3.4).

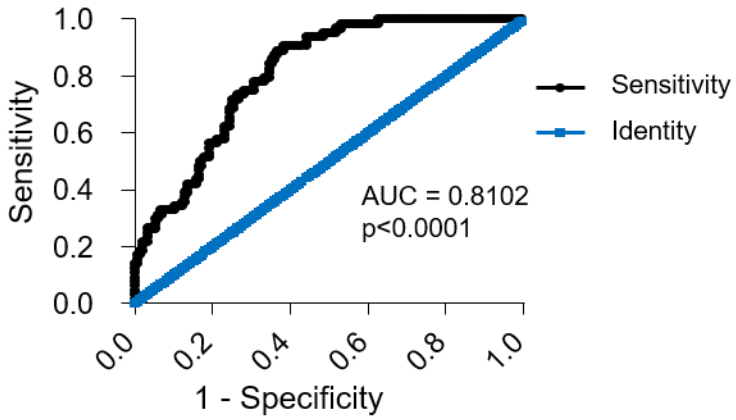


Figure 3.4 ROC curve analysis of the 2-miRNA signature. Receiver operating characteristic curve for the 2-miRNA signature when comparing benign meningioma to higher-grade. AUC and p value reported in figure, 95% confidence interval = 0.7534 to 0.8670 (n = 125).

3.1.5 Investigation of miR-15 family members levels in PDMN cells and meningioma cell lines

3.1.5.1 miR-15 family member expression in PDMN cells

I next investigated the expression levels of the miR-15 family members in our *in vitro* model of meningioma cells (25 WHO I PDMN, 9 WHO II PDMN, and the malignant KT21-MG1 cell line) to confirm the results obtained in tissues. This evaluation did not include WHO III PDMN cells due to lack of suitable samples and extreme difficulties culturing them *in vitro*. As calibrator, I used HMC (see section 2.4.3).

Similarly to what was observed in tumour tissues, the expression levels of miR-15a, -16-1, and -15b were unchanged between WHO I PDMN, WHO II PDMN, and KT21-MG1 cells (Fig. 3.5A, B, and C, respectively). However, there was a significant decrease in the expression levels of miR-497 and -195 in the malignant cell line compared to WHO I PDMN cells (-0.92 and -1.19 Log_{10} fold decrease, respectively). miR-497 was significantly downregulated in WHO II PDMN compared to WHO I PDMN cells, while miR-195 levels were unchanged when comparing WHO II and I PDMN cells (Fig. 3.5D and E, respectively).

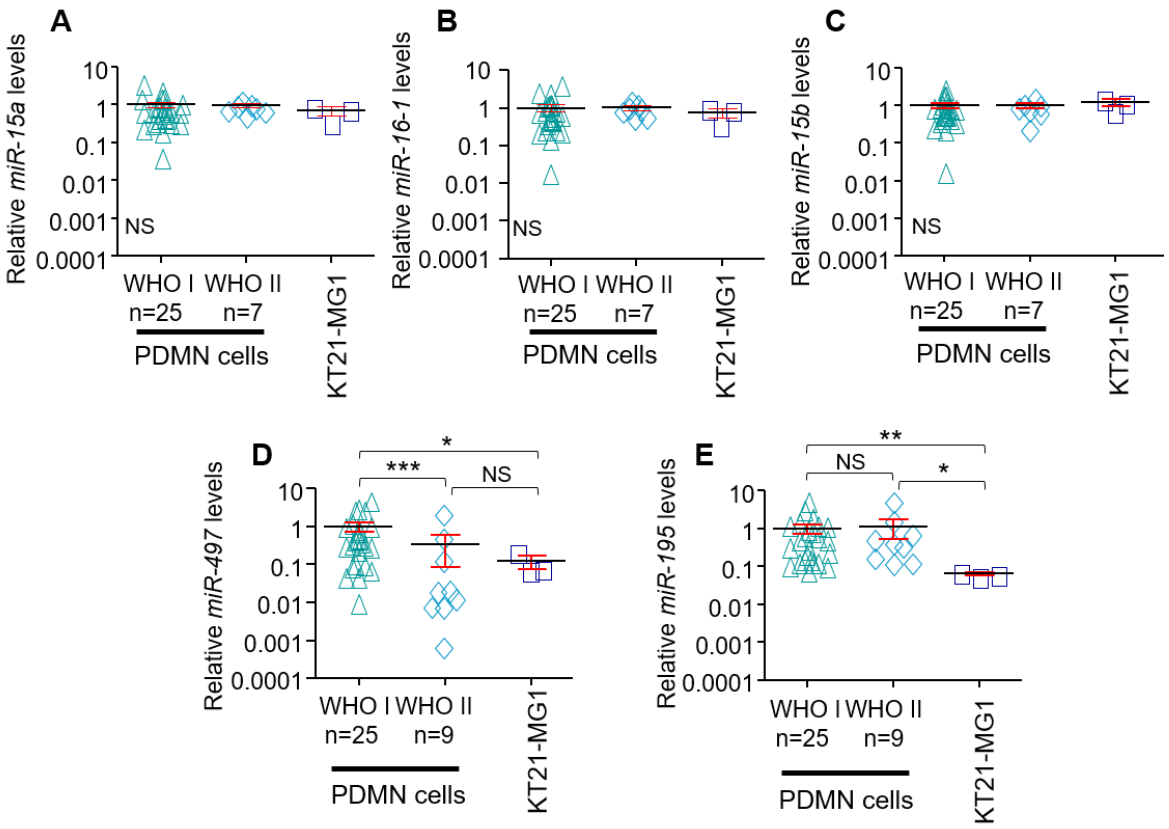


Figure 3.5 Gene expression analysis performed by RT-qPCR of the miR-15 family in WHO I and II PDMN cells. (A, B, C) Expression levels of miR-15a, -16-1, and -15b in WHO I (n = 25) and II PDMN (n = 7), and KT21-MG1 cells, respectively. (D, E) Expression levels of miR-497 and -195 in WHO I (n = 25) and II PDMN (n = 9) and KT21-MG1 cells, respectively. In all graphs expression levels were normalised against those in WHO I PDMN and shown as mean ± SEM in a Log_{10} scale. Three different passages of KT21-MG1 were used to ensure consistency. (NS = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$).

3.1.5.2 Expression levels of miR-15 family members in the benign Ben-Men-1 and malignant KT21-MG1 cell lines

Finally, I analysed the expression levels of the miR-15 family members in Ben-Men-1 and KT21-MG1 as all target validation studies were performed in these two cell lines (for consistency, I profiled each cell line at three different passages, using HMC as calibrator; see section 2.4.3). Indeed, a significant decrease of miR-15a and -16-1 (-0.74 and -0.82 Log_{10} fold decrease, respectively) was observed in KT21-MG1 cells when compared to Ben-Men-1, whilst miR-15b levels were unchanged (Fig. 3.6A, B, and C, respectively). Despite these results suggesting that Ben-Men-1 cells behave differently from PDMN (WHO I) cells, analysis of miR-497 and -195 demonstrated that these miRNAs are significantly downregulated in KT21-MG1 when compared to Ben-Men-1 (-1.46 and -1.42 Log_{10} fold decrease) (Fig. 3.6D and E, respectively).

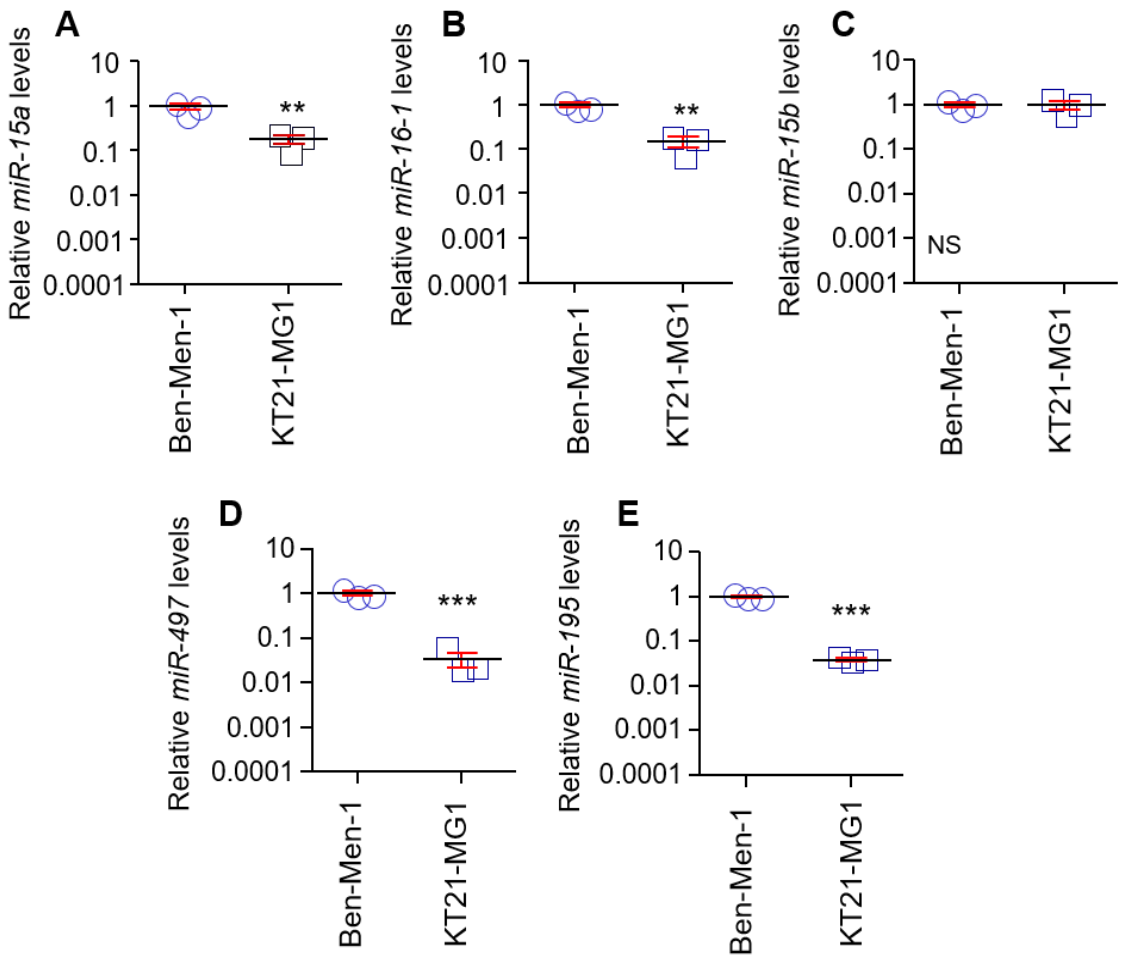


Figure 3.6 Gene expression analysis performed by RT-qPCR of the miR-15 family in Ben-Men-1 and KT21-MG1 meningioma cell lines. The expression levels of miR-15a and miR-16-1 are significantly downregulated in KT21-MG1, whilst levels of miR-15b remained unchanged (A, B, and C, respectively). Expression levels of both miR-497 and -195 were significantly downregulated in KT21-MG1 compared to Ben-Men-1 (D and E, respectively). All data were normalised to Ben-Men-1 and presented as mean ± SEM in a Log_{10} scale. For both cell lines each dot represents a different passage. (NS = not significant; ** = $p<0.01$; *** = $p<0.005$).

3.1.6 Discussion

The identification of new potential prognostic biomarkers for meningioma represented a priority of this study. Therefore, I first investigated whether protein and gene expression in our patient samples and *in vitro* model were in accordance with the previous literature showing that Cyclin D1 is overexpressed in meningioma tissues compared to healthy controls (Alama *et al.*, 2007; Milenkovic *et al.*, 2008).

Indeed, I observed that there was an increase in the expression levels of Cyclin D1 in meningioma cells and tissue, but the difference was only significant when comparing WHO I PDMN cells with HMC and not when comparing WHO I tumours to normal meninges control. This is likely due to the low number of tumour lysates analysed and the heterogeneity of meningioma (Gay *et al.*, 2011).

Nonetheless, this observation was in agreement with the previously published findings, suggesting that our model was suitable to be used in the project for the identification of potential biomarkers.

Cyclin D1 is not the only Cyclin with a role in the regulation of cell proliferation. Therefore, I also decided to investigate the expression levels of Cyclin D2 and D3 (which play a similar role as Cyclin D1 in the G_0/G_1 checkpoint), and E1 (G_1/S checkpoint) as parallel experiments suggested that these Cyclins share common translational regulators (Hydbring, Malumbres & Sicinski, 2016). In tumour lysates, all Cyclins tested were upregulated compared to normal meninges, but I did not observe a difference among the grades (Fig 3.1A). In WHO I PDMN cells I observed a trend to increase only in Cyclin D2 (n=11,

p=0.59 Fig. 3.1B). In this *in vitro* model, Cyclin D3 and E1 were unchanged compared to control. When analysing Cyclin expression in the available meningioma cell lines, I observed a dramatic increase in the expression levels of all Cyclins in KT21-MG1 compared to HMC and Ben-Men-1. Interestingly, an upregulation of CDK4, which binds to D-family Cyclins to exert their functional roles (Hydbring, Malumbres & Sicinski, 2016), was also observed in the malignant meningioma cell line compared to both Ben-Men-1 and HMC (Fig.3.2).

Overall, these results suggested that Cyclin D1 is the only pro-proliferative Cyclin biomarker showing altered expression in all the meningioma models considered in this study (Fig 3.1). *Cyclin D1, D2, D3, and E1* are all targeted by the miR-15 family members, as previously described (Hui *et al.*, 2013; Furuta *et al.*, 2013; Wei *et al.*, 2016). Indeed, investigation of the expression levels of all members of the miR-15 family revealed that one cluster, miR-497~195, is significantly downregulated in WHO III meningioma tissues and cells, compared to their benign counterparts, thus negatively correlating with the expression levels of their predicted targets (Fig. 3.3, 3.5, 3.6, respectively). This observation suggests that this miRNA cluster might be involved in the regulation of Cyclins expression in meningioma, and its members might represent potential molecular biomarkers for meningioma. Indeed, miR-497 and -195 proved to have a good diagnostic value when comparing benign and higher-grade (WHO II and III) meningioma tumours, both when considered independently from each other (Fig. 3.3F and G, respectively) and when considered as a 2-miRNA signature (Fig. 3.4).

miR-497 has previously been shown to have the potential to differentiate between WHO II and I meningioma when considered as part of a 4-miRNA signature (Ludwig *et al.*, 2015). Whist there are no such reports for miR-195 in meningioma, the function of the cluster has, however, been investigated in other tumours. For instance, miR-497~195 cluster has been shown to be downregulated in hepatocellular carcinoma (HCC) and its overexpression in HCC cell lines displayed growth-suppressive activities through induction of G_1 arrest due to the downregulation of its target mRNAs, among which the authors found *CDK6, CCNE1, CDC25A and CDK4* (Furuta *et al.*, 2013).

The findings reported in this section suggest that this miRNA cluster might have a similar function in meningioma, but this needs to be further investigated.

It is noteworthy that, with regards to PDMN cell lysates, I only had WHO I samples available due to the difficulties encountered when growing WHO II and III PDMN cells *in vitro*. By modifying the composition of cell culture media to include more FBS, I managed to expand some WHO II PDMN cells in culture. However, the cells grew very slowly and only for a limited number of passages (~2-4), therefore I chose not to collect lysates as quite a large amount of material was needed to include WHO II PDMN cells in all Western blot panels. Nonetheless, I was able to collect sufficient RNA from WHO II PDMN cells in order to include them in the gene expression screenings.

In summary, in this section I showed that the miR-497~195 cluster is significantly downregulated in malignant meningioma tissues and cells, and has a good diagnostic ability in discriminating between benign (WHO I) and high-grade (WHO II-III) meningioma patients, thus representing a potential biomarker.

Since my project is aimed at finding potential molecular biomarkers that can be found in blood circulation as well, in the next section I will present data concerning miR-497~195 expression and diagnostic ability in serum-derived exosomes.

3.2 Investigation of the expression levels of the miR-497~195 cluster in circulating exosomes

Introduction

microRNAs represent particularly interesting biomarkers as they can be found both in tissue and in the bloodstream as free circulating RNAs or carried in EVs, including exosomes (Kalluri, 2016). Exosomes are EVs of endocytic origin likely mirroring the physiologic or pathologic condition of the cell they originated from (Simons & Raposo, 2009). It has been shown that blood of cancer patients is twice as rich in exosomes as the blood of healthy individuals, highlighting their potential to be used as liquid biopsies (Kalluri, 2016).

In the previous section, I observed a significant downregulation of miR-497 and -195 in malignant compared to benign meningioma tissues and cells; moreover, I determined that they have a good diagnostic value in tissue, both when considered alone and as part of a 2-miRNA signature. Thus, in this one I assessed the expression levels of the miR-497~195 cluster in exosomes isolated from meningioma patients' serum samples and evaluated their diagnostic value through ROC curve analysis. Since isolation of exosomes from other EVs is continuously under debate (Thery *et al.*, 2006; Gyorgy *et al.*, 2011; Taylor & Shah, 2015), I evaluated the process of exosome harvesting by monitoring the expression of the exosome biomarkers CD63 and CD9 (membrane proteins of the tetraspanins family), and of Calnexin, GM130 (endoplasmic reticulum and Golgi markers, respectively) and GAPDH as positive cellular markers (Camacho, Guerrero & Marchetti, 2013).

Before investigating miR-497~195 cluster expression in serum-derived exosomes, I focussed the investigation on exosomes isolated from Ben-Men-1 and KT21-MG1 cell culture media, as they represent homogeneous and enriched preparations of exosomes derived from benign and malignant meningioma cells, respectively.

3.2.1 Quality control of the exosome preparations from cell culture media and meningioma patients' serum samples

Current methods to isolate exosomes do not guarantee the absence of other contaminating extracellular vesicles. Moreover, in both preparations from cell culture media and serum samples (see 2.1.3), cellular contaminations might be present, and they might affect the results of the qPCR analysis on miR-497~195 cluster expression. Therefore I decided to monitor the quality of the preparations by Western blot analysis of isolated exosomes. Both preparations from cell culture media and serum samples proved to be free from cellular contaminations, as verified by the lack of detection of the cellular markers in the lanes containing exosomes and the enrichment of the exosomal markers CD63 and CD9 (Fig. 3.7A and B, respectively).

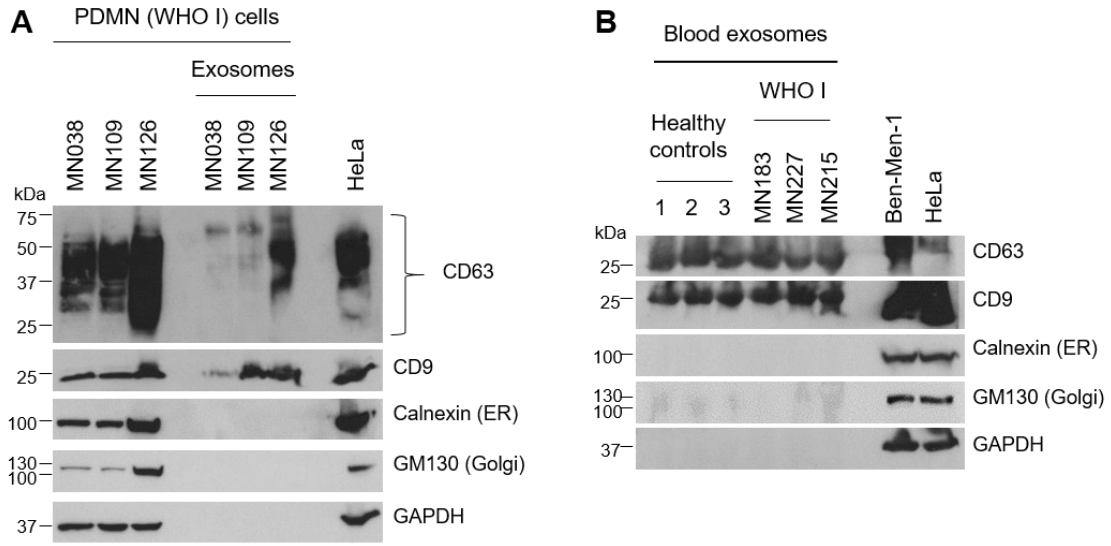


Figure 3.7 Representative Western blots showing assessment of exosome enrichments. (A) Three WHO I PDMN samples (MN038, MN109, MN126) and exosomes isolated from their cell culture media were used to assess exosome preparation quality through immunoblotting of CD63, CD9, Calnexin, GM130, and GAPDH. Lysate from HeLa cells was used as a positive control for antibody detection. (B) Exosomes isolated from serum samples of three healthy controls (1, 2, 3) and three meningioma patients (MN183, MN227, MN215) were used to assess serum exosome preparation quality. Lysates from Ben-Men-1 and HeLa cells were used as positive controls.

3.2.2 Investigation of the miR-497~195 cluster release through exosome cargo

The mechanism through which microRNAs are selected to be released from the cells into the extracellular space *via* exosomes is not completely understood, yet (Zhang *et al.*, 2015). Therefore, I first assessed our detection method of miR-497 and -195 *in vitro*, by analysing exosomes isolated from Ben-Men-1 and

KT21-MG1 cell culture media, which represent less complex and readily available starting materials compared to serum.

After exosome harvesting, total exosomal RNA was extracted, quantified, and the expression levels of the miR-497~195 cluster were measured by qPCR, using the snRNA U6 as an endogenous control and normalising miRNA expression using HMC to compute the comparative $\Delta\Delta C_T$ method (see sections 2.1.3, 2.4.2, and 2.4.3).

This analytical method was sufficient to detect and quantify both miR-497 and -195 in exosomes. It showed that expression levels of miR-497 and -195 were significantly lower in exosomes extracted from KT21-MG1 cell culture media compared to those isolated from Ben-Men-1 media (-2.66 and -2.60 Log_{10} fold decrease, respectively) (Fig. 3.8A and B, respectively). These results were consistent with those observed by profiling total RNA preparations from cells (Fig. 3.6D and E, respectively).

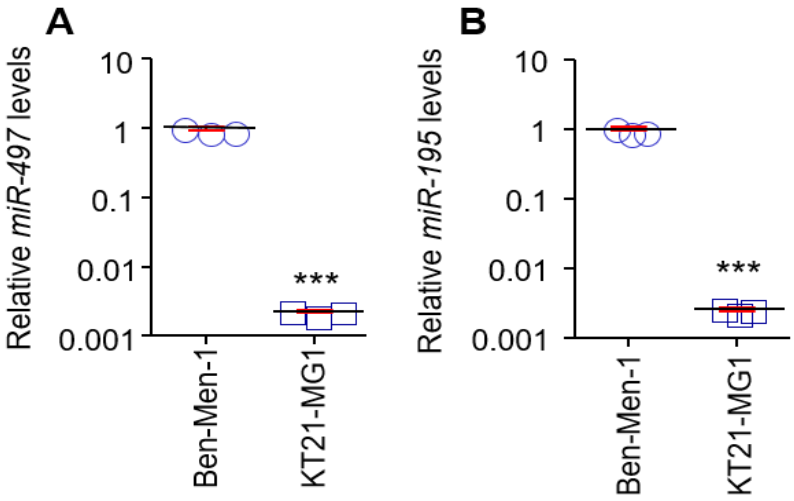


Figure 3.8 Analysis of miRNA cargo in exosomes isolated from cell culture media. Both miR-497 and -195 were detectable in exosomes extracted from Ben-Men-1 and KT21-MG1 cell culture media. Both miRNAs were significantly

downregulated in exosomes released from KT21-MG1 (n=3) compared to Ben-Men-1 (n=3), with those extracted from KT21-MG1 containing only ~0.22% of miR-497 and 0.25% of miR-195 compared to exosomes released from Ben-Men-1 (A and B, respectively). Data were normalised to the average expression in Ben-Men-1 and presented as mean ± SEM in a Log_{10} scale. (*** = $p<0.005$).

3.2.3 Investigation of the miR-497~195 cluster expression levels in meningioma patients' serum samples

Given the small number of blood samples received, it made sense to compare the expression levels of the two miRNA candidates in benign meningioma serum samples (24 WHO I patients) with higher-grade meningioma serum samples (15 WHO II and III patients grouped together), applying the protocol optimised *in vitro*. Blood samples were collected prior to surgery. The expression levels of miR-497 and -195 were normalised to those of the snRNA U6 and RNA extracted from exosomes derived from healthy volunteers' serum (n = 10) was used as a calibrator (see sections 2.1.3, 2.4.2, and 2.4.3).

Consistently with our results in tissues, both miR-497 and -195 were significantly downregulated in exosomes isolated from higher-grade meningioma patients (WHO II+III) when compared to exosomes isolated from WHO I patients (-1.27 and -0.30 Log_{10} fold decrease, respectively) (Fig. 3.9A and C, respectively). However, when assessing their diagnostic value through ROC analysis, as described in 2.4.5, miR-497 showed a higher AUC compared to miR-195 (0.8351 and 0.7632, respectively) (Fig. 3.9B and D, respectively).

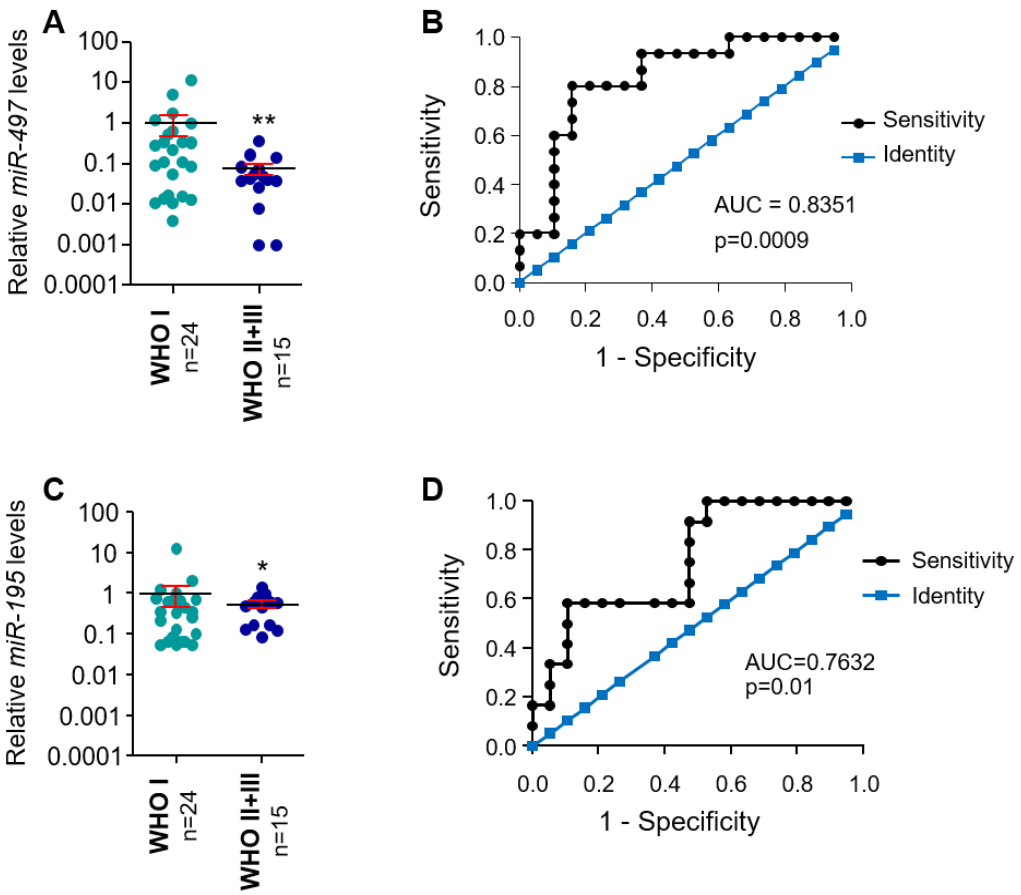


Figure 3.9 Analysis of miRNA cargo in exosomes isolated from meningioma patients' serum samples. Both miR-497 and -195 were downregulated in exosomes extracted from higher-grade meningioma patients' serum samples (WHO II+III, n = 15) compared to benign meningioma patients (WHO I, n = 24), with miR-497 showing a bigger decrease between the two groups than miR-195 (A and C, respectively). Data shown were normalised to the expression of miRNAs in WHO I meningioma patients' serum exosomes and presented as mean ± SEM in a Log_{10} scale. (* = $p<0.05$; ** = $p<0.01$). This difference was reflected in the ROC curve analysis where miR-497 (B) demonstrated a larger AUC compared to miR-195 (D) in serum exosomes (AUC and p value reported in figure; 95% confidence interval = 0.7426 to 0.9943 and 0.5446 to 0.9048, respectively), suggesting a better diagnostic value of miR-497.

As performed in tissues (Fig. 3.4), I decided to estimate the diagnostic value of the miRNA candidates when considered as a 2-miRNA signature in blood. The profile of the ROC curve obtained by combining the data for miR-497 and -195 expression in circulating exosomes of benign and high-grade (WHO II-III) meningioma tumours was not convincing, as showed by the AUC of 0.6695 (Fig. 3.10).

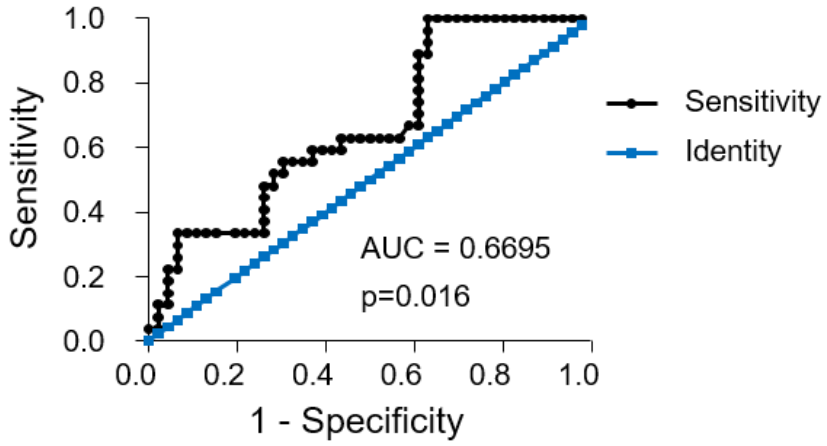


Figure 3.10 ROC curve analysis of the 2-miRNA signature in circulating exosomes. Receiver operating characteristic curve for the 2-miRNA signature (miR-497 and -195) when comparing benign meningioma (WHO I, n = 24) to higher grade (WHO II-III, n = 15). AUC and p value reported in figure, 95% confidence interval = 0.5438 to 0.7952.

3.2.4 Discussion

microRNAs can be released from cells into the extracellular space through EVs, including exosomes, suggesting that miRNA signatures in blood may serve as disease fingerprints and provide novel circulating biomarkers for cancer (Chen *et al.*, 2008; Mitchell *et al.*, 2008). Blood-based biomarkers have the important advantage of being minimally invasive, high-throughput and affordable (Zhi *et al.*, 2016).

A recent investigation on free-circulating miRNAs in meningioma led to the discovery that the levels of a 6-miRNA panel could distinguish meningioma patients from healthy controls with high sensitivity and specificity. Moreover, the expression signature of these miRNAs changed after tumour removal, proving to be potentially useful to monitor the effect of surgical resection in clinical practice (Zhi *et al.*, 2016). This study only investigated the expression levels of miRNAs extracted from whole serum. However, it has previously been shown that miRNAs in serum primarily exist inside exosomes and that using exosomal fractions increases the sensitivity of miRNA detection (Gallo *et al.*, 2012). Therefore, I decided to study the expression levels of the candidate miRNA cluster in exosomes isolated from WHO I, II and III meningioma patients' serum samples, in order to understand whether miR-497 and -195 could serve as circulating biomarkers for meningioma. Given the small number of WHO III serum samples received and the fact that higher-grade meningiomas (WHO II and III) pose a more challenging clinical management, WHO II and III patients' samples were grouped together for the purposes of this study, thus comparing the results between benign and higher-grade meningioma.

Before proceeding, I checked the quality of the exosome preparations, to make sure that they lacked contamination by cellular components and other EVs as has been previously demonstrated in some cases (Thery *et al.*, 2006; Gyorgy *et al.*, 2011; Taylor & Shah, 2015). Results suggested that no cellular debris were present in the preparations from either cell culture media or serum samples (Fig. 3.7A and B, respectively). In both cases, enrichment in the exosomal markers CD9 and CD63 was observed; however, detection of CD63 bands were different in exosomes from cell culture media from those extracted from serum (Fig. 3.7). This disparity is likely due to different glycosylation profiles of the protein in the two preparations. The predicted molecular weight of CD63 is 25 kDa (Ageberg & Lindmark, 2003; Kanada *et al.*, 2015), but it can be detected by Western blot analysis at the 32, 35, or 50 kDa markers depending on the degree of N-linked glycosylation (Tominaga *et al.*, 2014).

In line with my previous observations in tissues and cells (Fig. 3.3D and E, 3.5D and E and 3.6D and E, respectively), miR-497 and -195 were significantly downregulated in higher-grade meningioma serum samples compared to the benign group. As suggested from the ROC curve analysis, which quantifies how accurately a diagnostic test can discriminate between two populations, miR-497 seems to have a better diagnostic value compared to miR-195, implying that it could potentially be used as a circulating prognostic biomarker for higher-grade meningioma. In fact, a higher AUC suggests a higher sensitivity and specificity of the test in distinguishing between two groups; the maximum AUC = 1 means that the diagnostic test is perfect in differentiating between the two conditions, whereas the minimum AUC of 0.5 means that the diagnostic test is not better than chance (in this case the curve overlaps the line of identity) (Hajian-Tilaki, 2013).

When combined in a 2-miRNA signature, the ROC curve profile for miR-497 and -195 combined is very poor, likely due to the small sample size available (n = 39) and the variability observed (Fig. 3.9A and C).

Unfortunately, I was unable to determine whether changes in the expression levels of the two candidate miRNAs correlate with clinical outcome due to the relatively short nature of this project compared to the minimum time-frame to assess meningioma recurrence (established at five years). Therefore, first reliable data on recurrence for samples analysed here might be available from 2020.

Furthermore, collection of a larger number of blood samples, including serial samples from the same patient, is warranted in order to further investigate the efficacy of miR-497 and -195 in clinical practice.

The observation of lower levels of the miR-497~195 cluster in malignant meningioma tissues and cells, as well as in high-grade serum-derived exosomes, suggests a dysregulation at the transcriptional level. Therefore, in the next section, I will investigate the expression levels of GATA-4, a possible factor involved in the transcriptional regulation of the miR-497~195 cluster.

3.3 Identification of GATA-4 as a possible factor involved in the regulation of miR-497~195 cluster in meningioma

Introduction

miRNA-coding genes can be found in intergenic or intragenic locations throughout the genome. About 10% are situated within exons of protein-coding genes and, if encoded in the sense direction with the host gene, follow the same transcription pattern. It is plausible that miRNAs located within exons of cell lineage- or function-specific genes might contribute to the control of genetic networks according to the expected function of the host gene product (O'Carroll & Schaefer, 2013).

Approximately 40% of miRNA-coding genes are located within introns (Rodriguez *et al.*, 2004; Smalheiser *et al.*, 2008). Experimental and bioinformatics evidence has shown the ability of numerous intronic miRNAs to co-express with their host genes, even though initiation of RNA polymerase II-dependent transcription within an intron may prevent transcription and splicing of the protein-coding genes (Rodriguez *et al.*, 2004; Wang *et al.*, 2009).

The remaining 50% of miRNA-coding genes are located within the intergenic space; most of them are autonomously expressed and possess their own enhancer and promoter elements (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001; Corcoran *et al.*, 2009).

It has been shown that miRNA genes clustered within 0.1-50 kb from each other display common expression patterns (Baskerville & Bartel, 2005), whereas miRNA genes spaced more than 50 kb apart tend to express in a non-

coordinated fashion (Baskerville & Bartel, 2005). Since miR-497 and -195 are found in cluster on chromosome 17p13.1, it is likely that they share the same regulatory mechanism (Wei *et al.*, 2016), a hypothesis supported by the fact that they display similar expression levels in meningioma, as shown previously (sections 3.1 and 3.2).

In the previous sections I observed a significant downregulation of miR-497 and -195 in malignant meningioma cells and tissue, as well as in exosomes derived from high-grade (WHO II-III) meningioma patients, consistent with the hypothesis of a differential transcriptional regulation of these two miRNAs in high-grade compared to benign meningioma. Hence, in this section I am going to investigate whether this is true by determining the factor responsible for driving miR-497~195 cluster deregulation.

Various factors have been shown to be involved in the regulation of miR-497 and -195 expression levels. For example, expression levels of miR-195 in glioblastoma positively correlated with those of TGF-β1, and treatment of U87 glioblastoma cells with TGF-β1 led to an increase in the levels of miR-195 in a concentration dependent manner (Duan & Chen, 2016). However, TGF- β1 has been shown to be abundantly expressed in malignant meningioma (Gogineni *et al.*, 2012); as higher levels of TGF-β1 correlate with high levels of miR-195, this suggests that it might not be involved in the regulation of the miR-497~195 cluster in this tumour.

Interestingly, the transcription factor GATA binding protein 4 (GATA-4) has been shown to play a role in the regulation of some members belonging to the miR-15 family, such as miR-15b, miR-16 and miR-195. Overexpression of GATA-4 in rat mesenchymal stem cells led to a decrease in the expression of

these microRNAs and to increased resistance to ischemia (Yu *et al.*, 2013). Moreover, a bioinformatics analysis has predicted that there are binding sites for the GATAD2A and GATAD2B zinc finger domains in the promoter/enhancer region of the miR-497~195 cluster (Fishilevich *et al.*, 2017).

GATA-4 is a zinc finger transcription factor belonging to the GATA-4/5/6 family of transcription factors, implicated in the development and differentiation of embryonic stem cells, cardiovascular embryogenesis, and guidance of epithelia cell differentiation in adult (Lentjes *et al.*, 2016). It has been shown to be a survival factor for differentiated, postnatal cardiomyocytes, and an upstream activator of the anti-apoptotic genes *Bcl-xL* and *Bcl-2*, as well as *Cyclin D2* and *CDK4* (Aries *et al.*, 2004; Rojas *et al.*, 2008; Kobayashi *et al.*, 2010).

The *GATA-4* coding gene is located on chromosome 8p, which is a site frequently deleted in multiple tumour types, such as colorectal and oesophageal cancers (Derks *et al.*, 2006; Lin *et al.*, 2000). Alternatively, *GATA-4* can be downregulated via epigenetic silencing, such as hypoacetylation of histones H3 and H4 (Caslini *et al.*, 2006) and promoter CpG island hypermethylation, as observed in colorectal, gastric, oesophageal, lung, ovarian and HPV-driven oropharyngeal cancer, in glioblastoma multiforme, and in diffuse large B-cell lymphoma (Akiyama *et al.*, 2003; Guo *et al.*, 2004; Guo *et al.*, 2006; Wakana *et al.*, 2006; Pike *et al.*, 2008; Hellebrekers *et al.*, 2009; Agnihotri *et al.*, 2011; Kostareli *et al.*, 2013). These observations suggest that GATA-4 might play tumour suppressive roles in these disease settings.

However, *GATA-4* amplification has recently been described in certain gastric cancers, indicating a more oncogenic function (Chia *et al.*, 2015), and it has also been shown to promote the expression of the anti-apoptotic factor *Bcl-2*

and *Cyclin D2* in ovarian granulosa cell tumours, further supporting its role as an oncoprotein (Kyronlahti *et al.*, 2008; Anttonen *et al.*, 2014).

Interestingly, *GATA-4* expression patterns have never been investigated in meningioma. Thus, I decided to measure GATA-4 expression levels in meningioma tissue samples and also in the WHO I and II PDMN cells and meningioma cell lines through Western blot analysis, immunohistochemical staining, and qPCR, in order to calculate its potential diagnostic value. Furthermore, functional studies on GATA-4 were performed in the two cell lines, the benign Ben-Men-1 and malignant KT21-MG1, in order to further elucidate its roles in meningioma progression, all of which will be discussed in the following section.

3.3.1 Investigation of GATA-4 in meningioma

3.3.1.1 GATA-4 is upregulated in malignant meningioma tumours

Firstly, levels of *GATA-4* were evaluated by gene expression analysis, as described in section 2.4.4, uncovering a significant upregulation in malignant tumours when compared to WHO I (2.15 Log_{10} fold increase), while showing a trend to increase in WHO II samples compared to WHO I (2.00 Log_{10} fold increase, Fig. 3.11A). To investigate the diagnostic value of GATA-4 as a biomarker for malignant meningioma, I applied the ROC curve analysis by comparing WHO I versus WHO II-III tumours, as described in section 2.4.5. As depicted in Fig. 3.11B, the high value of the area under the curve (0.8319, $p<0.0001$) suggests that GATA-4 can be a potential diagnostic biomarker to

discriminate between WHO I (benign) and WHO II-III (higher-grade) tumours with high specificity and sensitivity.

To further support these results, I evaluated the protein levels of GATA-4 *via* Western blot analysis and immunohistochemical (IHC) staining of tumour tissues (Fig. 3.11C and D, respectively). Even though a significant increase at the protein level in WHO III samples compared to WHO I tumours was observed (1.32 fold change increase compared to WHO I, Fig. 3.11C), I was not able to confirm this result *via* Western blot analysis due to the difficulty to allocate an adequate amount of protein lysates from WHO II and III tissues surplus. However, it was possible to perform an extensive evaluation of GATA-4 in tissues using IHC. Staining was carried out by Leanne Kirk at the Cellular and Anatomical Pathology Department at Derriford Hospital, Plymouth, and was quantified by Dr David Hilton, as described in materials and methods section 2.3.2.

Supporting qPCR results, GATA-4 staining was stronger in malignant meningioma compared to benign samples and normal meningeal tissue (NMT). There was also a small increase in GATA-4 staining in atypical meningiomas compared to WHO I (Fig. 3.11D; for single score values see Table 3.3).

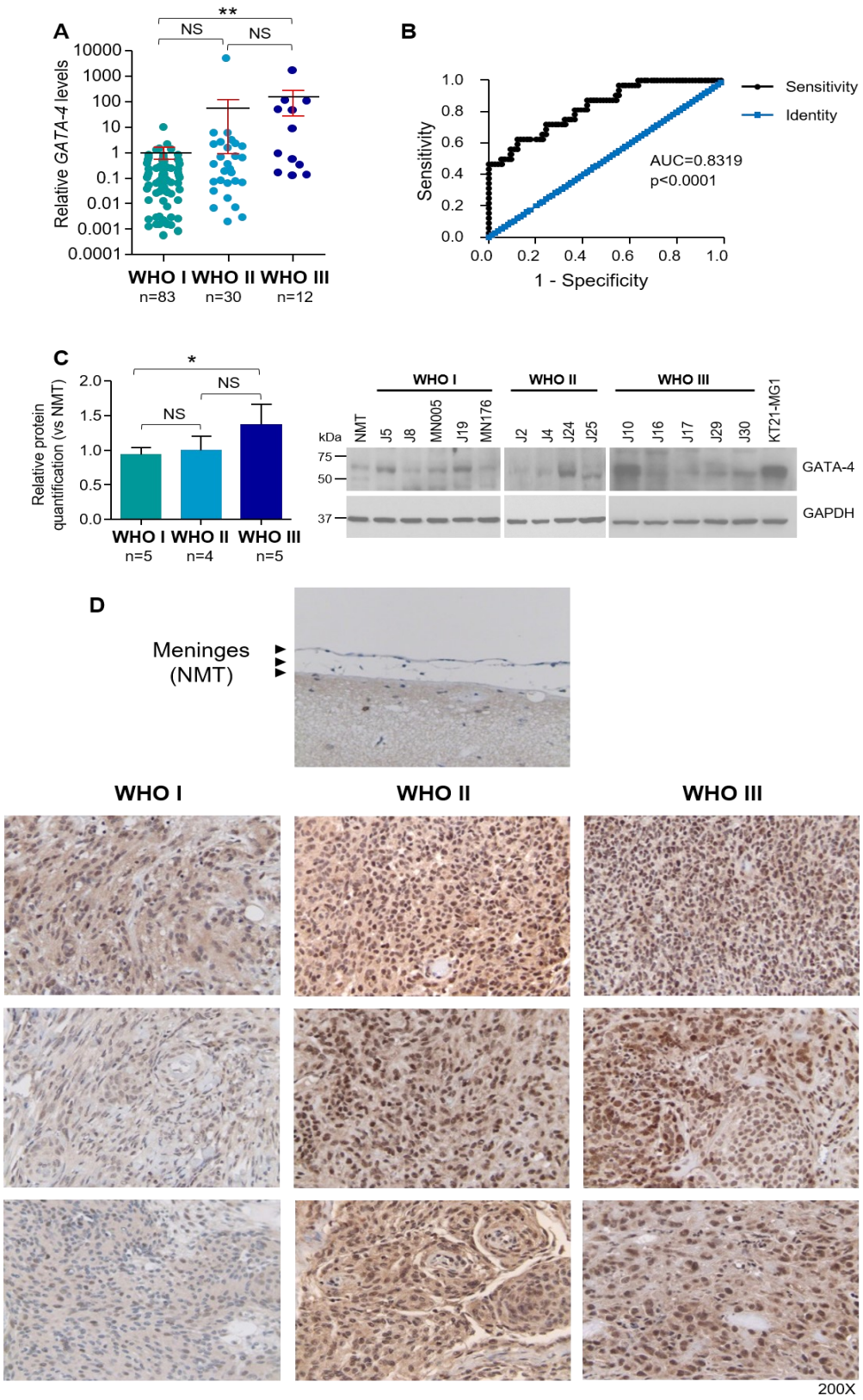


Figure 3.11 GATA-4 is upregulated in malignant meningioma tumour samples. (A) Gene expression analysis performed by RT-qPCR of *GATA-4* showing a significant increase in grade III tissues (n = 12) compared to benign

meningioma samples (n = 83). Data were normalised to the average expression in WHO I tissues and shown as mean ± SEM in a Log_{10} scale. (B) Receiver operating characteristic curve based on the expression of *GATA-4* at the transcriptional level (AUC and p value reported in figure; 95% confidence interval = 0.7492 to 0.9146). (C) Relative quantification and immunoblot of GATA-4 showing a significant increase in WHO III samples (n = 5) when normalised to the mean of WHO I samples (n = 5). (D) Representative images showing GATA-4 immunohistochemical staining in WHO II and III meningiomas compared to WHO I and normal meninges (NMT, see black arrows), at 200X magnification. Single score values reported in Table 3.3. (NS = not significant; * = $p<0.05$; ** = $p<0.01$).

WHO I	GATA-4	WHO II	GATA-4	WHO III	GATA-4	Meninges	GATA-4
J5	2	J1	1	J9	2	NM1	1
J6	2	J2	1	J10	3	NM8	1
J14	1	J3	2	J11	3	NM9	0
J15	1	J4	1	J12	3	NM11	1
MN005	2	MN020	3	NRG3M1	3	NM14	1
NRG1M8	2	J13	3	NRG3M6	3	NM3	0
G1M34	2	MN018	3	NRG3M7	2	NM23	1
NRG1M7	1	MN001	2	NRG3M11	3	NM6	1
G1M42	2	NRG2M7	2	NRG3M15	3	NM5	0
G1M45	2	NRG2M14	1	NRG3M16	3	NM16	1

Table 3.3 Single IHC score values for GATA-4 intensity. 0 = negative staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

3.3.1.2 Expression of GATA-4 in meningioma, *in vitro*

3.3.1.2.1 GATA-4 is upregulated in the malignant cell line KT21-MG1 compared to WHO I and II PDMN cells

Having shown that *GATA-4* is overexpressed in malignant meningioma tissues, I then studied its expression levels in WHO I and II PDMN cells and in the malignant cell line KT21-MG1.

In agreement with the above results in tissues (Fig. 3.11), *GATA-4* was significantly overexpressed in KT21-MG1 cells compared to WHO I PDMN cells, both at the transcriptional (3.37 Log_{10} fold increase) and protein levels (Fig. 3.12A and B, respectively). Gene expression analysis (see 2.4.4) did not reveal a difference in *GATA-4* mRNA expression between WHO I (n = 25) and WHO II (n = 9) PDMN cells (Fig. 3.12A).

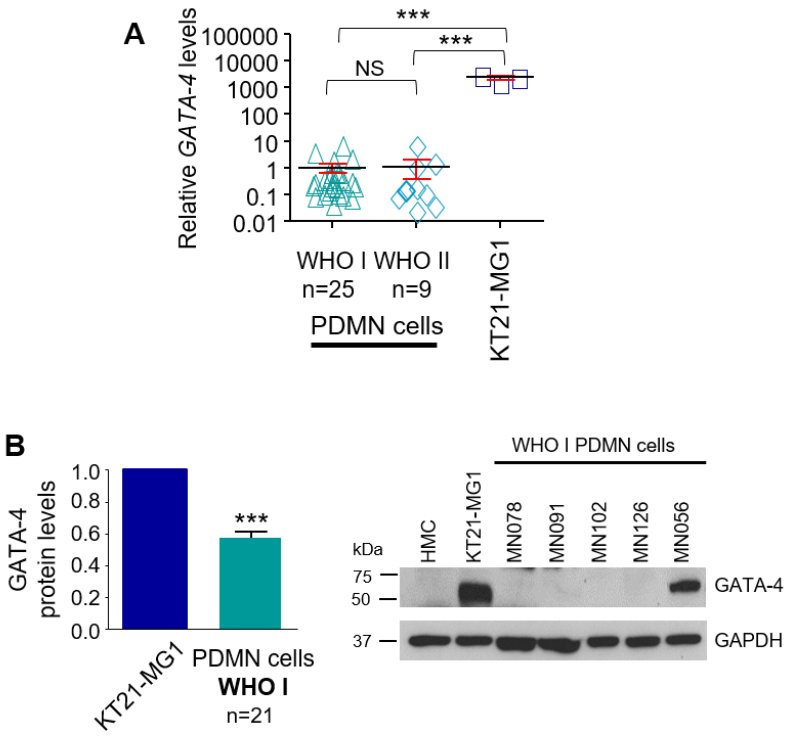


Figure 3.12 Gene expression and Western blot analysis reveal GATA-4 upregulation in KT21-MG1 compared to WHO I PDMN cells. (A) Significant upregulation of *GATA-4* is demonstrated at the transcriptional level in KT21-MG1 compared to WHO I PDMN cells (n = 25; 3.37 Log_{10} fold increase). No differences in the mRNA level were observed when comparing WHO I and WHO II PDMN cells (n = 9). Data presented have been normalised to the average expression in WHO I PDMN and shown as mean ± SEM in a Log_{10} scale. (B) Relative quantification and representative Western blot of GATA-4 in normal human meningeal cells (HMC), KT21-MG1 and WHO I PDMN cells (n = 21). (NS = not significant; *** = $p<0.005$).

3.3.1.2.2 GATA-4 is overexpressed in KT21-MG1 when compared to Ben-Men-1 cells

I also compared the expression of GATA-4 between KT21-MG1 and Ben-Men-1 cells and, as expected, GATA-4 was significantly upregulated in the malignant compared to the benign cell line, as observed through Western blot and gene expression analysis (Fig. 3.13A and B, respectively; see 2.4.4 for gene expression analysis protocol). Notably, GATA-4 shows a 50.40 fold change increase at the protein level in KT21-MG1 compared to Ben-Men-1 and a 4.25 Log_{10} fold increase at the mRNA level.

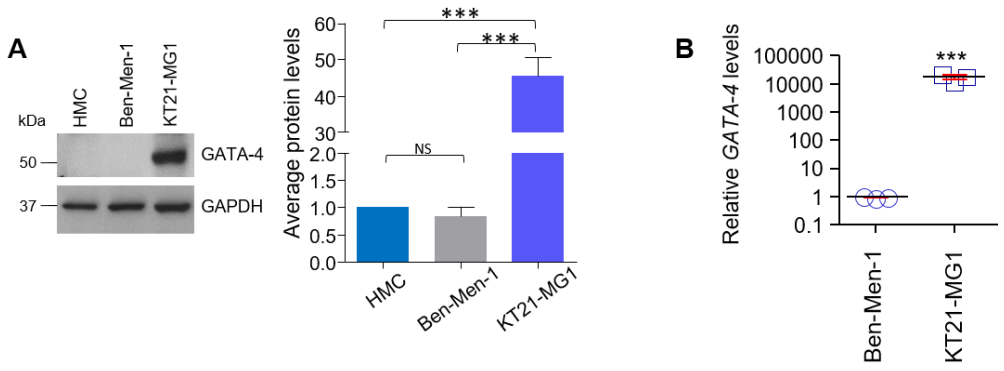


Figure 3.13 GATA-4 is overexpressed in KT21-MG1 compared to Ben-Men-1. (A) Representative Western blot of GATA-4 in human meningeal cells (HMC), benign Ben-Men-1 and malignant KT21-MG1 cells. One out of three Western blots is shown. GATA-4 shows a 45.87 fold increase in KT21-MG1 when compared with HMC, and a 50.40 fold increase when compared with Ben-Men-1. (B) Gene expression analysis performed by RT-qPCR of *GATA-4* in KT21-MG1 and Ben-Men-1 cell lines. Each dot represents a different passage of the same cell line to ensure consistency. Data shown have been normalised against the average expression of *GATA-4* in Ben-Men-1 cells and presented as mean ± SEM in a Log_{10} scale. (NS = not significant; *** = $p<0.005$).

Since GATA-4 is part of the GATA-4/5/6 family of transcription factors, and needs to interact with FOG2 to exert its functional roles (Crispino *et al.*, 2001), I went on to evaluate the expression of GATA-5, GATA-6 and FOG2 in Ben-Men-1 and KT21-MG1 cells *via* Western blot analysis. There seems to be no difference in the expression of these factors between Ben-Men-1 and KT21-MG1 cell lines (Fig. 3.14), however, as only one passage per cell line was run, I was unable to perform a statistical analysis on these data.

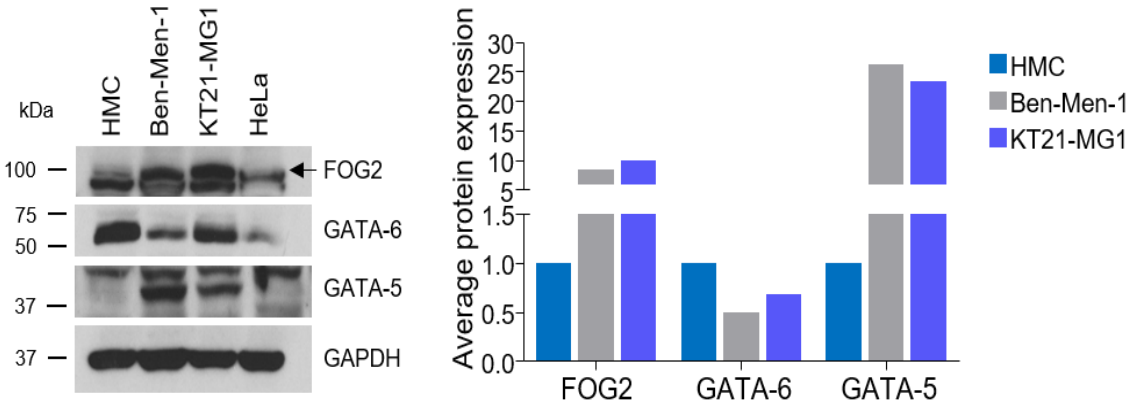


Figure 3.14 FOG2, GATA-6 and GATA-5 expression levels in Ben-Men-1 and KT21-MG1 cells. Western blot for FOG2, GATA-6 and GATA-5 in HMC, Ben-Men-1 and KT21-MG1 cells. One HeLa cell lysate has been used as a control. (FOG2 upper band).

3.3.2 Lentiviral-mediated transduction of *GATA-4* in Ben-Men-1 cells leads to downregulation of the miR-497~195 cluster increasing cell viability

To investigate whether GATA-4 can play a pro-proliferative role in meningioma and correlates with the miR-497~195 cluster, I infected Ben-Men-1 cells with lentiviral particles carrying a p-Lenti-GIII-*GATA-4* vector, as described in materials and methods section 2.2.1. GFP-coding lentiviral particles (p-Lenti-GIII-GFP) were used as control. Ben-Men-1 cells represent a suitable model to perform this analysis as they express high levels of both miR-497 and -195 and low levels of GATA-4 (see Fig. 3.6D and E, and 3.13A and B, respectively).

To monitor transfection and vector expression efficiency, I first measured levels of GATA-4 in Lenti-*GATA-4* and Lenti-GFP transfected cells, both at the transcriptional and protein levels. Gene expression analysis (see 2.4.4) confirmed that, after transduction, mRNA levels of *GATA-4* increased in Ben-Men-1 (1792.77 fold change), compared to control (Fig. 3.15A). After demonstrating *GATA-4* overexpression at the mRNA level, increased protein levels were also detected (13.99 fold change) (Fig. 3.15B). To further demonstrate *GATA-4* infection efficiency, expression levels of Bcl-2, a known transcriptional target of GATA-4 (Kobayashi *et al.*, 2010), were monitored. Indeed, upon *GATA-4* overexpression, Bcl-2 expression increased both at the protein and transcriptional levels (1.52 and 1.1 fold change, respectively) (Fig. 3.15B and C, respectively).

qPCR analysis revealed that after overexpression of *GATA-4* in Ben-Men-1 cells, expression levels of the miR-497~195 cluster significantly decreased

(around 90% decrease for miR-497 and 70% decrease for miR-195), while miR-15a, -16-1 and -15b showed a trend to decrease (Fig. 3.15D), .

Previous studies (Furuta *et al.*, 2013; Wei *et al.*, 2016; Yin *et al.*, 2017) revealed that miR-497 and -195 are involved in the regulation of *Cyclin D1, D2, D3, and E1* expression levels. Therefore, after overexpression of *GATA-4* in Ben-Men-1 cells, I investigated the effects of miR-497~195 downregulation on Cyclins expression levels.

RT-qPCR analysis highlighted a significant increase in *Cyclin D1, D2 and E1* expression levels (1.2, 16.53, and 2.31 fold change, respectively), and a trend to decrease of *Cyclin D3* (Fig. 3.15E). Consequently, Cyclins were upregulated at the protein level, which led to an increase of phospho-RB levels, a known target of the activity of the Cyclin D1-CDK4/6 complex (Hydbring, Malumbres & Sicinski, 2016) (Fig. 3.15F). It is noteworthy that, even though a non-significant decrease of *Cyclin D3* was observed through qPCR (Fig. 3.15E), its protein levels increased following *GATA-4* overexpression (Fig. 3.15F).

Through ATP assay (see section 2.2.2.1), which measures the amount of intracellular ATP, thus indicating the metabolic activity of the cell, which can be linked to cell viability, I observed a significant increase in Ben-Men-1 viability upon lentiviral-mediated *GATA-4* overexpression (around 20% more, as measured by ATP assay) (Fig. 3.15G).

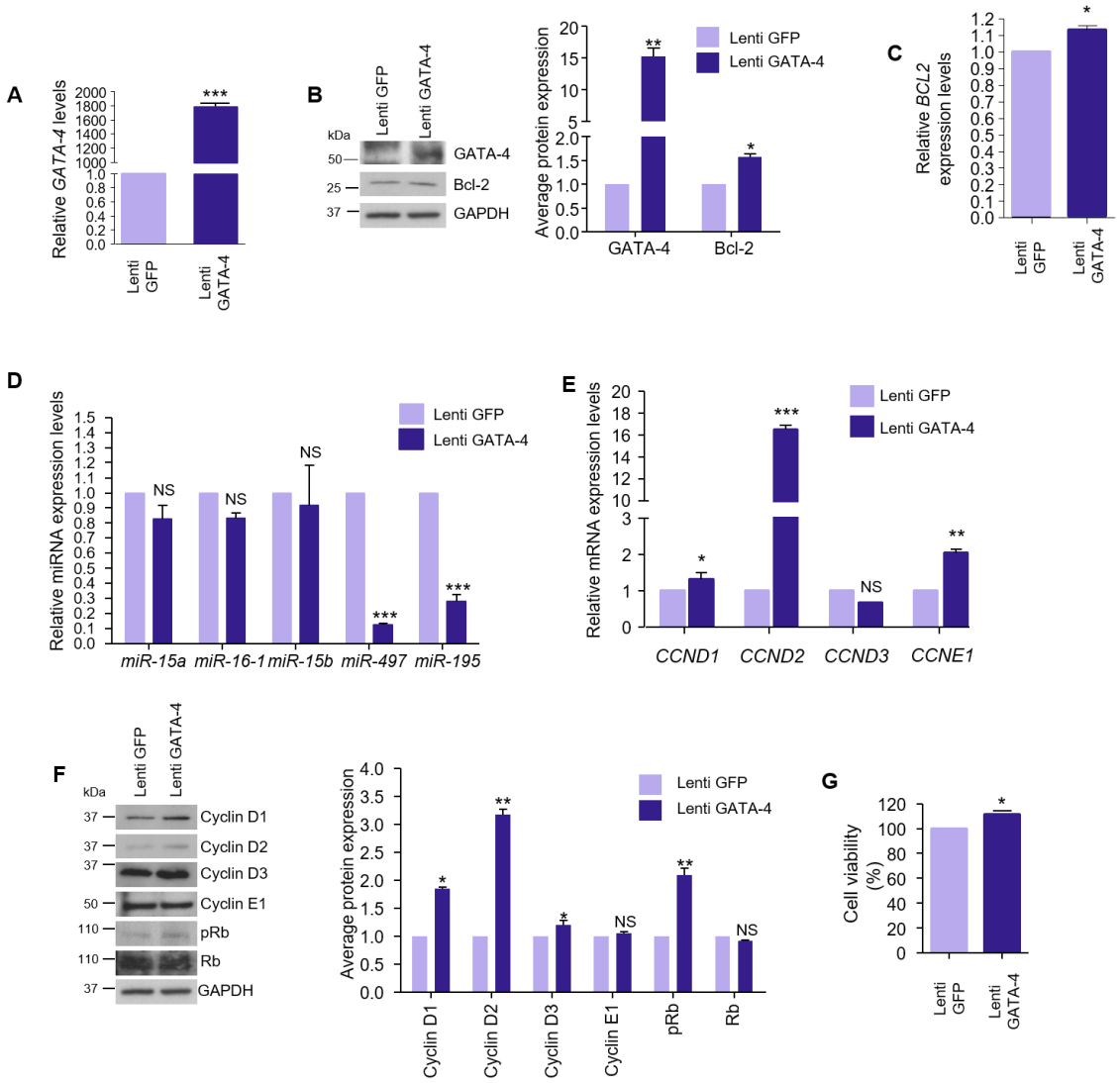


Figure 3.15 Effects of lentiviral-mediated *GATA-4* transduction in Ben-Men-1 cells. (A) Gene expression analysis performed by qPCR showed that the lenti *GATA-4* vector was efficiently expressed, leading to an increase in the levels of *GATA-4* mRNA. Data shown have been normalised to GFP-transfected cells and shown as mean ± SEM. (B) Representative Western blot and relative protein quantification showing increased GATA-4 and Bcl-2 protein expression following transduction. (C) Gene expression analysis showing increase of *Bcl-2* expression following lentiviral-mediated *GATA-4* transduction. *GAPDH* was used as an endogenous control. Data were normalised to the control transfected cells and shown as mean ± SEM. (D) qPCR analysis on

miR-15a, -16-1, -15b, -497 and -195 expression levels upon *GATA-4* transduction. Data have been normalised to the expression levels of p-Lenti-GIII-GFP transfected cells and shown as mean ± SEM. snRNA U6 was used as an endogenous control. (E) Gene expression analysis of *Cyclin D1, D2, D3 and E1* performed by qPCR following *GATA-4* overexpression. *GAPDH* was used as an endogenous control. Data were normalised to the control transfected cells and shown as mean ± SEM. (F) Representative Western blot and relative protein quantification showing increased Cyclin D1, D2, D3, E1, and phospho-RB protein expression in p-Lenti-GIII-*GATA-4* transfected cells when compared to control. (G) ATP assay demonstrates *GATA-4* overexpression increases Ben-Men-1 cell viability compared to control transfected cells. Data reported as mean ± SEM. (NS = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$). n = 3.

3.3.3 Lentiviral-mediated RNA interference of *GATA-4* in KT21-MG1 cells leads to upregulation of the miR-497~195 cluster decreasing cell viability

To further demonstrate the relationship between GATA-4 and the miR-497~195 cluster in meningioma, I performed a lentiviral-mediated RNA interference of *GATA-4* in the malignant KT21-MG1 cell line, which endogenously expresses low levels of miR-497 and -195 and high levels of GATA-4 (see Fig. 3.6D and E, and 3.13A and B, respectively), using short hairpin RNA (shRNA) sequences, as described in 2.2.1.

As expected, infection of KT21-MG1 cells with shGATA-4 lentiviral particles led to a 60% decrease in the expression levels of *GATA-4* mRNA, when compared

to scramble control (Fig. 3.16A). In parallel, GATA-4 was downregulated at the protein level by 92% in shGATA-4 infected cells, compared to scramble (Fig. 3.16B). To further monitor *GATA-4* knockdown efficiency, expression levels of Bcl-2 were monitored, demonstrating a decrease both at the protein (~56% decrease) and at the transcriptional level (~10% decrease) when compared to scramble control (Fig. 3.16B and C, respectively).

These results confirmed efficient *GATA-4* knockdown in KT21-MG1 cells, thus prompting me to monitor the expression levels of the miR-497~195 cluster, confirming its significant upregulation when compared to the scramble control (2.05 and 1.54 fold change, respectively), while miR-15a, -16-1 and -15b showed a trend to increase following *GATA-4* RNA interference (Fig. 3.16D).

Interestingly, *GATA-4* knockdown, probably through increased levels of miR-497 and -195, reduced *Cyclin D1, D2, D3, and E1* mRNAs levels (~35%, 40%, 37%, and 42% decrease, respectively, for *CCND1, CCND2, CCND3, and CCNE1*), compared to scramble (Fig. 3.16E).

Western blot analysis of Cyclin D1, D2, D3, and E1 consistently showed a decrease of their protein levels (~62%, 24%, 20%, and 17% decrease compared to scramble control, respectively), together with a 29% decrease in the levels of phospho-RB (Fig. 3.16F). I also measured shGATA-4-infected KT21-MG1 viability via ATP assay, and observed a significant decrease compared to scramble-infected cells (~30%) (Fig. 3.16G).

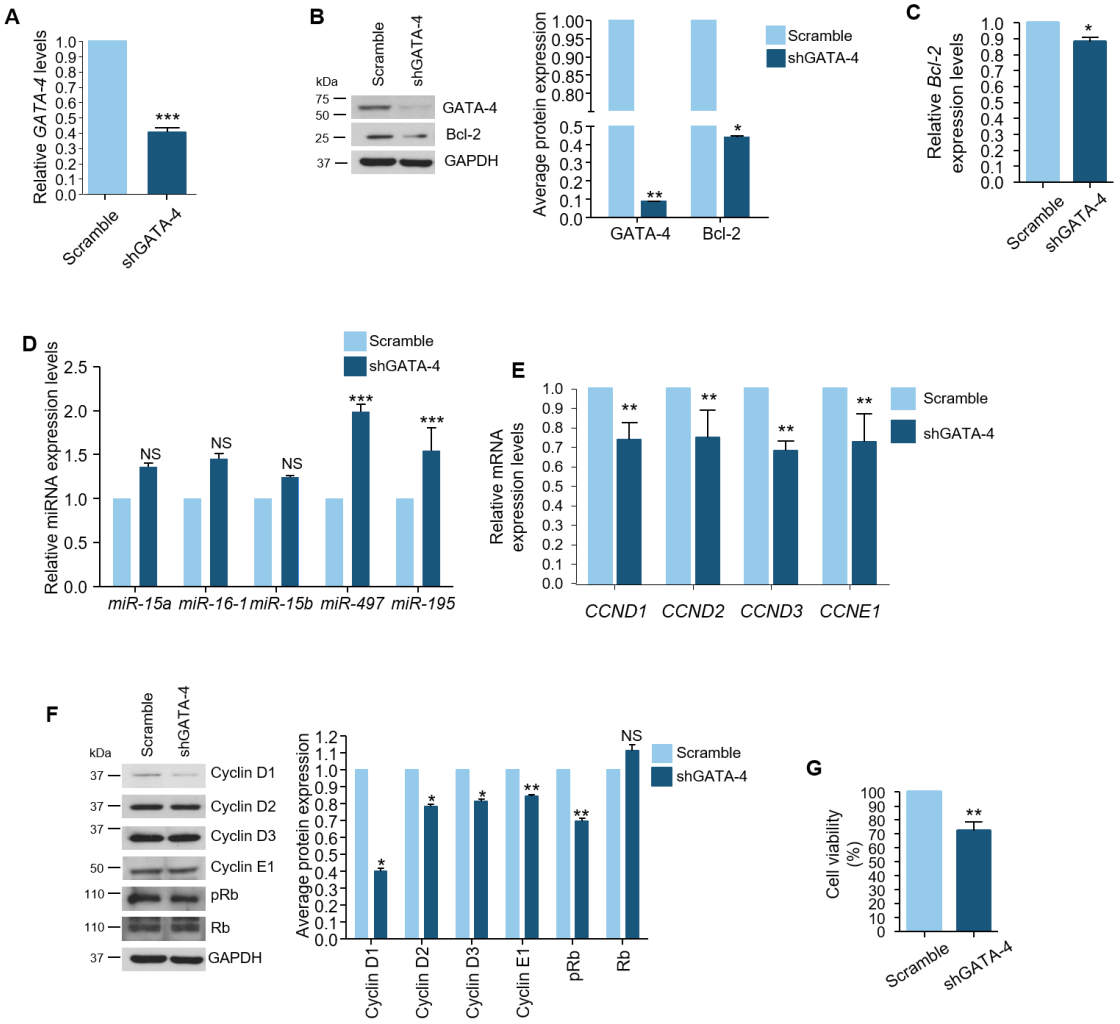


Figure 3.16 Effects of lentiviral-mediated interference of *GATA-4* in malignant KT21-MG1 cells. (A) Gene expression analysis revealed that *GATA-4* is significantly downregulated in shGATA-4 infected cells compared to scramble control (n = 3). Data shown have been normalised to the expression levels in the scramble control and reported as mean ± SEM. GAPDH was used as a reference gene. (B) Representative Western blot and relative protein quantification demonstrating the expression levels of GATA-4 and its transcriptional target Bcl-2 following *GATA-4* knockdown. (C) Gene expression analysis of *Bcl-2* expression levels following *GATA-4* knockdown. Data have been normalised to the scramble control and reported as mean ± SEM. *GAPDH* mRNA expression was used as endogenous control. (D) qPCR analysis of the

miR-15 family following *GATA-4* knockdown. The snRNA U6 was used as an endogenous control. Data shown have been normalised to the expression of the miRNA of interest in the scramble control and reported as mean ± SEM. (E) Gene expression analysis of *Cyclin D1, D2, D3, and E1* expression levels following lentiviral-mediated RNA interference of *GATA-4* in KT21-MG1 cells. The expression levels of *GAPDH* mRNA were used as endogenous control. Data have been normalised to the average expression in the scramble control and reported as mean ± SEM. (F) Representative Western blot and relative protein quantification of Cyclin D1, D2, D3, E1, and phospho-RB protein levels following *GATA-4* RNA interference. (G) ATP assay revealed a decrease in cell viability upon transfection with shGATA-4 lentiviral particles. Data have been normalised to the cell viability in scramble control and shown as mean ± SEM. (NS = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$). n = 3.

3.3.3.1 The miR-497~195 cluster regulates Cyclins expression and is involved in the feedback regulation of GATA-4

To further show the involvement of the miR-497~195 cluster in the regulation of Cyclin D1, D2, D3 and E1 expression levels in meningioma, as suggested by previous literature (Furuta *et al.*, 2013; Wei *et al.*, 2016; Yin *et al.*, 2017), I investigated the effects of lentiviral-mediated miR-195 transduction in KT21-MG1 cells.

To monitor transduction efficiency, I first measured the expression levels of miR-195 on transduced cells; as shown in Fig. 3.17A, upon infection of KT21-MG1 with LentimiRa-GFP-hsa-*miR-195-5p*, which codes for the mature miR-

195, there was an increase in the expression levels of miR-195 when compared to cells infected with Lenti-III-miR-GFP control (3.31 fold change).

miR-195 overexpression led to a decrease of *Cyclin D1, D2, D3, and E1* mRNA levels, as confirmed by gene expression analysis (~40%, 56%,14%, and 40% decrease, respectively; see 2.4.4) (Fig. 3.17B).

Furthermore, a feedback regulatory loop between a microRNA and its targets is often observed in various cell settings, both in physiological and pathological conditions (Quan *et al.*, 2019; Yang *et al.*, 2019; Hou *et al.*, 2019; He *et al.*, 2019). Moreover, the search performed through open-access databases revealed that the 3' UTR of *GATA-4* mRNA harbours a potential binding site for the miR-15 family (TargetScanHuman7.2 algorithm; Agarwal *et al.*, 2015) (Table 3.4). However, thus far, *GATA-4* has never been experimentally validated as a target of the miR-15 family.

miRNA	Position in the UTR	seed match	context++ score	context++ score percentile	weighted context++ score	conserved branch length	Pct
Conserved sites							
hsa-miR-15a-5p	856-862	7mer-m8	-0.19	86	-0.19	3.194	0.5
hsa-miR-497-5p	856-862	7mer-m8	-0.18	86	-0.18	3.194	0.5
hsa-miR-15b-5p	856-862	7mer-m8	-0.18	85	-0.18	3.194	0.5
hsa-miR-195-5p	856-862	7mer-m8	-0.18	85	-0.18	3.194	0.5
hsa-miR-16-5p	856-862	7mer-m8	-0.17	84	-0.17	3.194	0.5

Table 3.4 miR-15 family binding sites on the 3' UTR of *GATA-4* mRNA.

As predicted, overexpression of miR-195 in KT21-MG1 cells led to decreased *GATA-4* mRNA levels when compared to control (16% decrease, demonstrated

using qPCR) (Fig. 3.17C). This resulted in decreased levels of GATA-4 protein (55% decrease), as confirmed through Western blot analysis (Fig. 3.17D).

Interestingly, I observed a significant increase in the expression of miR-497 (8.89 fold change) (Fig. 3.17E), likely due to the lower levels of GATA-4 in KT21-MG1 cells overexpressing miR-195, when compared to control.

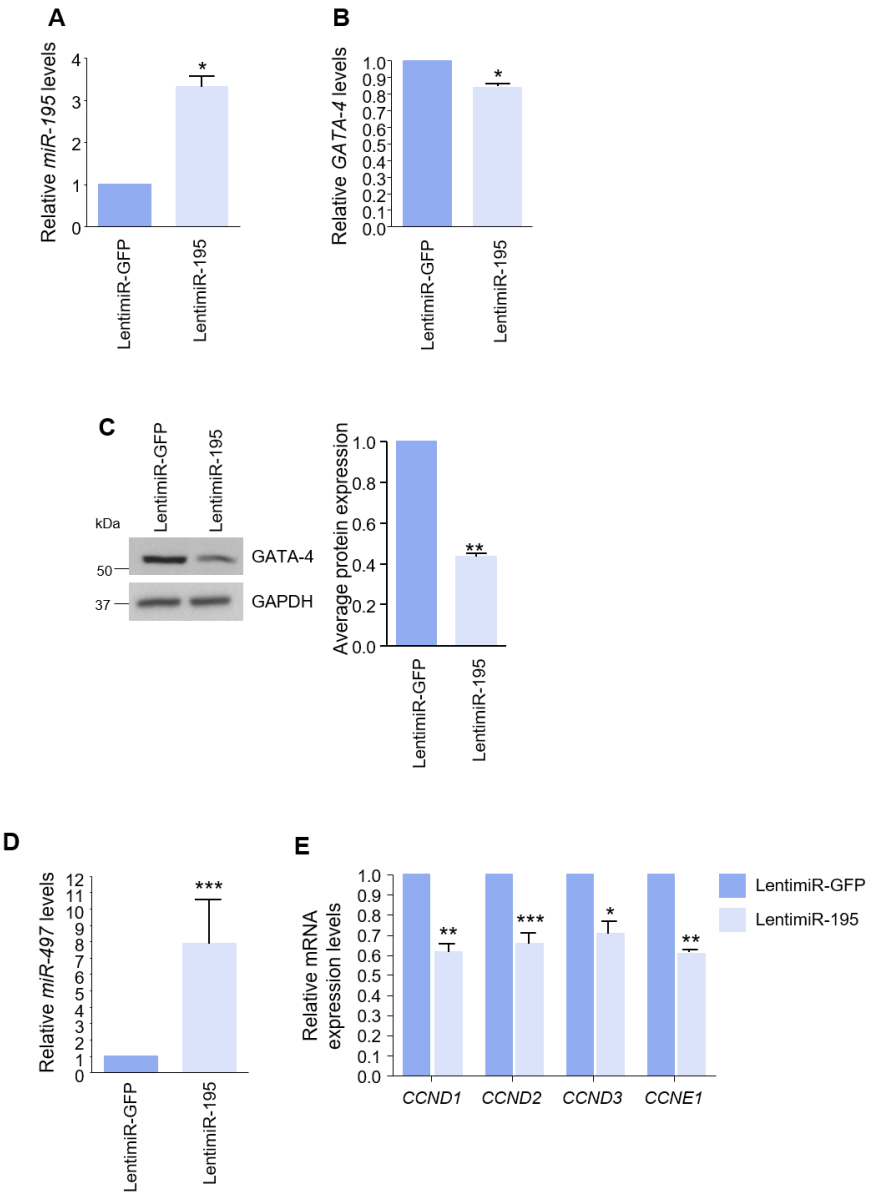


Figure 3.17 Effects of lentiviral-mediated miR-195 transduction in KT21-MG1 cells. (A) qPCR analysis confirmed miR-195 upregulation upon infection of KT21-MG1 cells with LentimiRa-GFP-hsa-miR-*195-5p*, when compared with

LentimiR-GFP-infected cells. The snRNA U6 was used as an endogenous control. Data have been normalised to LentimiR-GFP-infected cells and shown as mean ± SEM. (B) Gene expression analysis performed by qPCR showed decreased expression of *Cyclin D1, D2, D3, and E1* mRNA upon miR-195 overexpression. The expression levels of *GAPDH* mRNA were used as endogenous control. Data have been normalised to the average *Cyclin D1, D2, D3, and E1* expression in control cells and shown as mean ± SEM. (C) Gene expression analysis of *GATA-4* mRNA expression following miR-195 overexpression in KT21-MG1. The expression levels of *GAPDH* mRNA were used as endogenous control. Data have been normalised to control cells and shown as mean ± SEM. (D) Representative Western blot showing decreased expression of GATA-4 at the protein level after overexpression of miR-195. (E) qPCR revealed overexpression of miR-497 after infection with LentimiRa-GFP-hsa-miR-*195-5p*. The expression levels of the snRNA U6 have been used as endogenous control. Data have been normalised to control cells and shown as mean ± SEM. (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$). n = 3.

3.3.4 Discussion

3.3.4.1 GATA-4 is upregulated in malignant meningioma

GATA-4 is a zinc finger transcription factor, which plays important roles during embryogenesis and development (Lentjes *et al.*, 2016). Moreover, it has been shown to be upregulated following cardiac stress to allow survival of cardiomyocytes; its cardio-protective effects involve upregulation of the anti-apoptotic genes *Bcl-xL* and *Bcl-2*, but it has also been shown to regulate *Cyclin D2* and *CDK4* expression (Aries *et al.*, 2004; Rojas *et al.*, 2008; Kobayashi *et al.*, 2010).

Its role in cancer is not completely understood yet, as it seems to have tissue-specific functions. In fact, it has been found to be downregulated in multiple malignancies, (Lin *et al.*, 2000; Guo *et al.*, 2004; Derks *et al.*, 2006; Guo *et al.*, 2006; Wakana *et al.*, 2006; Pike *et al.*, 2008; Agnihotri *et al.*, 2011; Kostareli *et al.*, 2013) suggesting that GATA-4 might play tumour suppressive roles.

However, *GATA-4* overexpression has recently been described in certain gastric cancers and in ovarian granulosa cell tumours, indicating a more oncogenic function (Kyronlahti *et al.*, 2008; Anttonen *et al.*, 2014; Chia *et al.*, 2015).

Since it has been shown to be potentially involved in the regulation of the miR-15 family in rat mesenchymal stem cells (Yu *et al.*, 2013), and bioinformatics analysis predicted the presence of GATAD2A and GATAD2B binding sites in the promoter/enhancer region of the miR-497~195 cluster (Fishilevich *et al.*, 2017), I decided to investigate its expression levels in meningioma to

understand whether GATA-4 demonstrated a negative correlation with the expression levels of the miR-15 family, and, if so, to perform functional studies in order to test this hypothesis.

Indeed, I found that GATA-4 is significantly overexpressed in malignant meningioma cells and tissues, in which the expression levels of the miR-15 family, especially of the miR-497~195 cluster, were considerably lower compared to those in benign meningioma samples. This observation suggested an involvement of GATA-4 in the negative regulation of the miR-497~195 cluster.

Although the levels of both miR-497 and -195 are significantly lower in atypical compared to benign meningiomas, GATA-4 levels showed a trend to increase in WHO II compared to WHO I tumours. However, the difference is non-significant and I observed a high variability in its expression levels in WHO II tissues.

This could be related to the limitations of the applied histopathological criteria. Brain invasion has been introduced as a histological criterion that can alone suffice for diagnosing an atypical meningioma (Louis *et al.*, 2016), but these tumours might show a clinical course similar to WHO I. Moreover, meningioma frequently shows discordance between histological grade and biological behaviour (Milenkovic *et al.*, 2008), and some grade II tumours can progress to grade III tumours, thus it could be that these samples still retain some atypical features, sufficient to grade them as WHO II and explain the variability observed (Fig. 3.2 D and E, and 3.11A, C, and D, respectively).

I hypothesised that GATA-4 could be a novel biomarker for malignant meningioma, as it proved to have a good diagnostic value in ROC curve analysis when comparing WHO I and WHO II-III meningioma samples.

GATA-4 is part of the GATA-4/5/6 family of transcription factors (TFs), and the three TFs have closely related functions during development (Lentjes *et al.*, 2016). Therefore, I assessed the expression levels of GATA-5 and -6 in Ben-Men-1 and KT21-MG1 cells to understand whether they followed a similar expression pattern. FOG2 was also included in the investigation, as it has been shown to be a key interacting partner for GATA-4 in order to exert its functional roles (Crispino *et al.*, 2001; Carter *et al.*, 2014).

Although only one Western blot has been run for the analysis, and biological replicates are needed to confirm this findings, preliminary assessment of FOG2, GATA-5 and GATA-6 in Ben-Men-1 and KT21-MG1 cells did not lead to the detection of differences in the expression of these factors between the benign and the malignant meningioma cell lines (Fig. 3.14). Interestingly, FOG2 and GATA-5 seem to be upregulated in both samples when compared to the HMC whereas GATA-6 seems to be downregulated in both Ben-Men-1 and KT21-MG1 cells compared to normal meningeal cells (Fig. 3.14).

These three factors have been associated with cancer as well, but their roles are not completely understood and only a few reports are available to date.

FOG2 is often found to be co-expressed with GATA-4, especially in sex cord-derived ovarian tumours (Laitinen *et al.*, 2000; Virgone *et al.*, 2012).

GATA-5 has been shown to be downregulated in cancer due to CpG island hypermethylation, which is a predictor of poor clinical outcome in renal cell carcinoma (Peters *et al.*, 2014a; Peters *et al.*, 2014b). Moreover, the observation that GATA-5 overexpression in a colorectal cancer model led to decreased colony formation, proliferation, migration, invasion, and anchorage-

independent growth further supports the idea that GATA-5 functions as a tumour suppressor (Hellebrekers *et al.*, 2009).

GATA-6 expression has been shown to positively correlate with metastasis and negatively with overall survival in breast cancer patients (Song *et al.*, 2015); on the contrary, GATA-6 immunoreactivity proved to be stronger in malignant mesothelioma than in metastatic pleural adenoma, and it was observed that patients expressing GATA-6 had a significantly better prognosis than those with GATA-6 downregulation (Lindholm *et al.*, 2009).

Since the expression of these factors was only assessed in Ben-Men-1 and KT21-MG1 cell lines by Western blot analysis, but not in tissues and in primary cells, nor any functional assays were undertaken, I have insufficient data to draw conclusions on the function of these factors in meningioma. From what I observed, expression of FOG2, GATA-5 and GATA-6 is independent from that of GATA-4. Further investigations are needed in order to understand fully whether they play a role in meningioma genesis or progression.

3.3.4.2 GATA-4 controls the expression of the miR-497~195 cluster in meningioma

To test the hypothesis that GATA-4 could play a role in the regulation of the miR-497~195 cluster in meningioma, I overexpressed *GATA-4* in Ben-Men-1 and knocked it down in KT21-MG1 cells to monitor changes in the expression of miR-15 family members.

Lentiviral-mediated *GATA-4* overexpression in Ben-Men-1 showed that there is a significant decrease in the expression levels of the miR-497~195 cluster, which ultimately leads to increased Cyclin D1, D2, D3, and E1 expression (Fig. 3.15). As a consequence, I observed increased phosphorylation of the retinoblastoma protein (RB), and a significant increase in cell viability.

Conversely, lentiviral-mediated knockdown of *GATA-4* led to upregulation of the miR-497~195 cluster, ultimately leading to a decrease in the expression levels of Cyclin D1, D2, D3, and E1, a reduction in the phosphorylation status of RB, and a significant decrease in cell viability (Fig. 3.16).

It is interesting to note that, even though variations were observed in the expression levels of the other members of the miR-15 family, *i.e.* miR-15a, -16-1, and -15b, following modulation of *GATA-4* expression, these did not reach statistical significance in either Ben-Men-1 or KT21-MG1 cells.

This observation is in line with their expression levels in meningioma tissues and WHO I-II PDMN cells, in which miR-15a, -15b and -16-1 did not show variability due to tumour grade, suggesting a regulation which does not depend on GATA-4 expression.

The major limitation of this study is that I can show that GATA-4 regulates the expression of the miR-497~195 cluster but not the mechanism involved. In fact, as the promoter/enhancer region of the miRNA cluster is not known, but can only be predicted *via* bioinformatics analysis based on its genomic location, I could not prove a direct binding of GATA-4 on the miR-497~195 cluster promoter. Therefore, I cannot confidently demonstrate whether the effects observed following lentiviral-mediated overexpression or RNA interference of

GATA-4 are directly due to increased/decreased binding of GATA-4 to the promoter of the miRNA cluster or to indirect mechanisms.

Moreover, throughout this section, I used an ATP assay to assess viability of the cells after transduction. This assay measures the intracellular levels of ATP, thus indicating the metabolic status of a cell, which can be indirectly related to cell viability. However, this measure might be biased by the presence of quiescent cells, therefore more reliable assays to measure cell viability and death should be used to confirm the data presented here.

3.3.4.3 Possible feedback regulatory loop between GATA-4 and the miR-497~195 cluster

Since a feedback regulatory loop is often observed between a microRNA and the proteins coded by its targeted mRNAs (Quan *et al.*, 2019; Yang *et al.*, 2019; Hou *et al.*, 2019; He *et al.*, 2019), and the 3' UTR of *GATA-4* is predicted to harbour binding sites for miR-15 family members, I next evaluated the effect of miR-195 overexpression in KT21-MG1 cells on GATA-4.

Indeed, miR-195 overexpression led to a decrease in GATA-4 expression and, interestingly, an increase in the expression levels of miR-497 as well. Since miR-497 and -195 belong to the same miRNA family and share the same seed sequence, it might be argued that detection of increased levels of miR-497 was due to low primer specificity and cross-detection during qPCR; however, this is unlikely as the sequences flanking the seed region of the two mature miRNAs are different and I used the 3p fragment of the pre-miRNAs as a specific primer during miRNA amplification through RT-PCR.

As expected, miR-195 overexpression and consequent GATA-4 downregulation, led to a decrease of *Cyclin D1, D2, D3 and E1* mRNAs.

Targeting of *GATA-4* mRNA by miR-195 should be further confirmed with a reporter experiment in which the 3' UTR of *GATA-4* should be cloned upstream of a reporter gene such as *GFP* or *luciferase* to prove a direct binding of the miRNA on its target sequence.

Furthermore, it would be interesting to investigate whether miR-497 overexpression in KT21-MG1 leads to similar results and whether simultaneous overexpression of miR-195 and -497 increases the rate of GATA-4 downregulation.

Overall, these data suggest that GATA-4 plays an oncogenic function in meningioma, by negatively modulating the expression of the miR-497~195 cluster, which has been shown to have tumour suppressive activity (Li *et al.*, 2011; Furuta *et al.*, 2013; Hui *et al.*, 2013; Du *et al.*, 2015a; Luo *et al.*, 2016).

Moreover, I speculate that GATA-4 can be considered a specific biomarker and a possible novel therapeutic target for malignant meningioma.

3.4 The small molecule compound NSC140905, a selective GATA-4 inhibitor, decreases viability of malignant meningioma cells and shows potential to increase sensitivity to the CDK4 inhibitor PD0332991

Introduction

Transcription factors are proteins that bind to the DNA helix at specific regulatory sequences in order to activate or inhibit transcription through a transactivation or transrepression domain (Lambert *et al.*, 2018), and there is more and more evidence on their involvement in human pathologies (An *et al.*, 2016; Lambert *et al.*, 2018).

Transcription factors, as many other proteins, are spatially, temporally, and sequentially expressed in tissues during development, cell renewal or differentiation processes. Therefore, any modification of their expression may result in major deregulation of cell homeostasis, leading to pathologies, such as neurodegenerative disorders (Srinivasan & Lahiri, 2015; Tiwari & Pal, 2017; Gomez-Pastor, Burchfiel & Thiele, 2018; Hwang & Zukin, 2018; Dinkova-Kostova, Kostov & Kazantsev, 2018), diabetes and cardiac diseases (Polvani *et al.*, 2016; Lilly, Lacaud & Kouskoff, 2017), and cancer (Schito & Semenza, 2016; Sur & Taipale, 2016; Link & Fernandez-Marcos, 2017; Chen *et al.*, 2017a).

Although transcription factors are clearly associated with oncogenic addiction (Bradner, Hnisz & Young, 2017), only a small number is currently targeted in clinic, because they have been long considered as ‘undruggable’ targets (Yan & Higgins, 2013). Only a better knowledge of their precise functions and the

mechanisms through which they exert their roles has opened new possibilities for therapeutic intervention (Lambert *et al.*, 2018).

Various strategies are available to directly or indirectly target transcription factors:

- Targeting the transcription factor at the expression level, for instance using HDAC inhibitors such as valproic acid and Vorinostat (Garcia-Manero *et al.*, 2008; Fredly, Gjertsen & Bruserud, 2013);
- Directing the transcription factors for degradation, using compounds such as bortezomib (Desterro, Rodriguez & Hay, 2000; Liu *et al.*, 2016), mebendazole (Walf-Vorderwulbecke *et al.*, 2018) or honokiol (Zhou *et al.*, 2017);
- Inhibiting the ability of the transcription factors to interact with protein co-factors (including themselves in case they need to form homodimers to become functional), using protein-protein interaction inhibitors (PPIi), such as Nutlin-3 (Lambert *et al.*, 2018);
- Targeting the ligand-binding or DNA-binding domains of the transcription factors, for example using all-*trans*-retinoic acid (ATRA) (de The *et al.*, 1990) or ellipticine (Duffy, Synnott & Crown, 2017), respectively.

In 3.3 I observed that GATA-4 is significantly overexpressed in malignant compared to benign meningioma, and that it is involved in the regulation of the miR-497~195 cluster; therefore, in this section I am going to prove its potential as a therapeutic target.

To date, there are no drugs available to specifically target GATA-4. However, some compounds able to bind to the C-terminal zinc finger domain of GATA-4 and inhibit its interactions with the DNA have been identified (El-Hachem &

Nemer, 2011). It is known that if the structure of the C-terminal zinc finger is destabilized, GATA-4 will lose its binding affinity, as this domain is sufficient and essential for binding to the GATA consensus sequence (A/TGATAA/G) (El-Hachem & Nemer, 2011). In this study, authors performed a compound screening using *in vitro* cell-based assays, showing that selected molecules can specifically and efficiently inhibit GATA-4 activity by inhibiting its interaction with the DNA in a dose-dependent manner, thus preventing transcriptional activation of its downstream targets.

Since NSC140905 (2-(1,3-benzodioxol-5-ylmethyl)butanedioic acid) (Fig. 3.18) was the most effective in reducing GATA-4 binding to the DNA even at the lowest tested dose (60% reduction at 50 μM), reducing the expression of GATA-4 targets by 35% in HeLa cells, I questioned whether it could inhibit GATA-4 in KT21-MG1 cells.

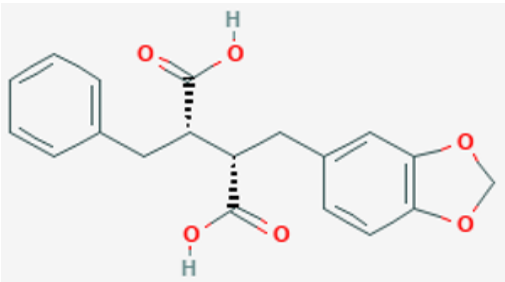


Figure 3.18 NSC140905 chemical structure. NSC140905 is a derivative of succinic acid, a known compound with zinc chelating properties (El-Hachem & Nemer, 2011).

3.4.1 NSC140905 decreases malignant meningioma cell viability through upregulation of the miR-497~195 cluster

The first step of this *in vitro* pharmacological evaluation was to determine the optimal range of concentrations of NSC140905, and for this purpose I titrated five concentrations (12.5 - 25 - 50 - 100 μM, including vehicle - 0.1% DMSO - as control) at three time points (24, 48, and 72 hours), as described in 2.2.2.

I observed that 50 μM of NSC140905 led to a significant reduction of cell viability at 24 and 48 hours (~20% reduction), whereas 100 μM of the molecule led to a significant reduction in cell viability at all the three time points considered (~30% reduction at 24, 48 and 72 hours, Fig. 3.19, in line with what I observed following lentiviral-mediated *GATA-4* RNA interference, see Fig. 3.16G). Considering these results, I decided to focus all following studies on the 24 hours time point.

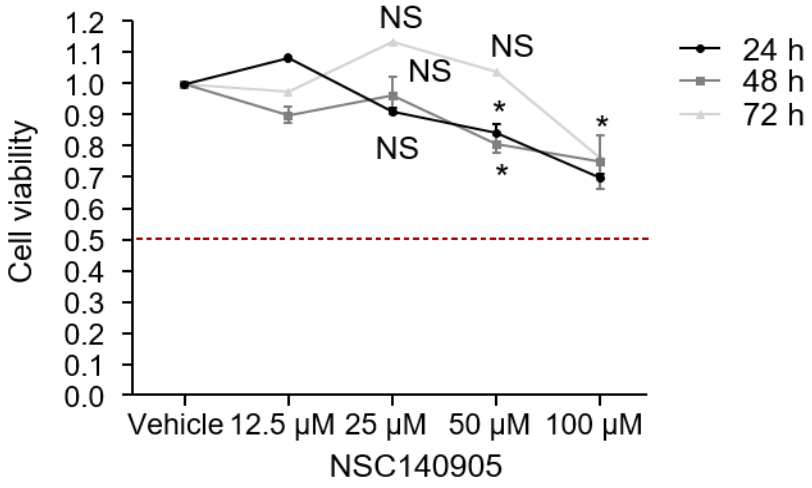


Figure 3.19 NSC140905 titration on KT21-MG1 cells. Cell viability was measured through ATP assay after 24, 48 and 72 hours of treatment with NSC140905. 0.1% DMSO was used as a vehicle. (NS = not significant; * = $p<0.05$).

Notably, a range of concentrations between 50 and 100 μM could lead to potential side effects on non-cancerous cells. Therefore, I tested the effects of NSC140905 in normal human meningeal cells (HMC) and three different primary Schwann cells (MOS; all Schwann cells used in this section are S100 positive, as shown by immunofluorescence, Fig. 3.20).

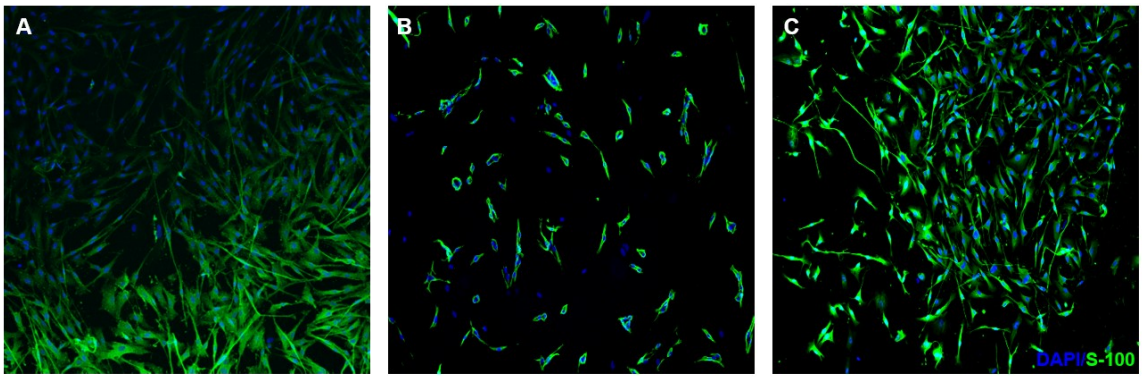


Figure 3.20 Schwann cells positivity for S100. S100 positivity of the Schwann cell samples used to assess NSC140905 activity on normal primary cells, MOS0318 (A), MOS0319 (B), and MOS0718 (C). DAPI was used to stain the nuclei. Schwann cell cultures were considered as free from fibroblast contamination when >90% positivity for S100 was observed. 10X magnification. Schwann cell isolation and immunofluorescence were carried out by Dr Emanuela Ercolano.

As reported in Fig. 3.21A, no signal for GATA-4 was detected by Western blot in neither HMC nor Schwann (MOS) cells. Moreover, treatment of HMC and Schwann cells with increasing doses of NSC140905 did not lead to decreased cell viability, whereas it was effective on KT21-MG1 at the highest doses tested (11% and 31% decrease at 50 and 100 μM, respectively, when compared to vehicle, 0.1% DMSO), as mentioned above, suggesting that the NSC140905

molecule did not affect viability in HMC and MOS cells, even at relatively high doses effective in KT21-MG1 cells (Fig.3.21B).

Once it was established that NSC140905 decreases viability of malignant meningioma cells (Fig. 3.21B), I assayed the expression levels of the miR-497~195 cluster in KT21-MG1 cells after treatment with the GATA-4 inhibitor. Treatment with 12.5 μM NSC140905 led to a non-significant increase in miR-497 expression, whereas miR-195 levels remained unchanged. However, at all the other concentrations tested, I detected a significant increase of the expression levels of both miRNAs (Fig. 3.21C).

Since the maximum effect on the miR-497~195 cluster expression was observed at 50 and 100 μM, I investigated the inhibition of GATA-4 transcriptional activity by monitoring the expression levels of *Bcl-2* at these concentrations. As expected, I observed a 26% decrease in the protein levels of Bcl-2 at both concentrations (Fig. 3.21D).

With regards to Cyclins, a 28% decrease in the expression levels of Cyclin D1 at 50 μM was seen when compared to vehicle, with a further reduction at 100 μM (68% decrease compared to 0.1% DMSO-treated cells). A similar effect was observed on the expression levels of Cyclin D2 (13% and 43% reduction at 50 and 100 μM, respectively, Fig. 3.21E).

To further confirm inhibition of cell cycle progression, I monitored the phosphorylation status of RB; as expected, a significant decrease in phospho-RB levels at both 50 and 100 μM was observed (~33% and 43% decrease, respectively, Fig. 3.21E).

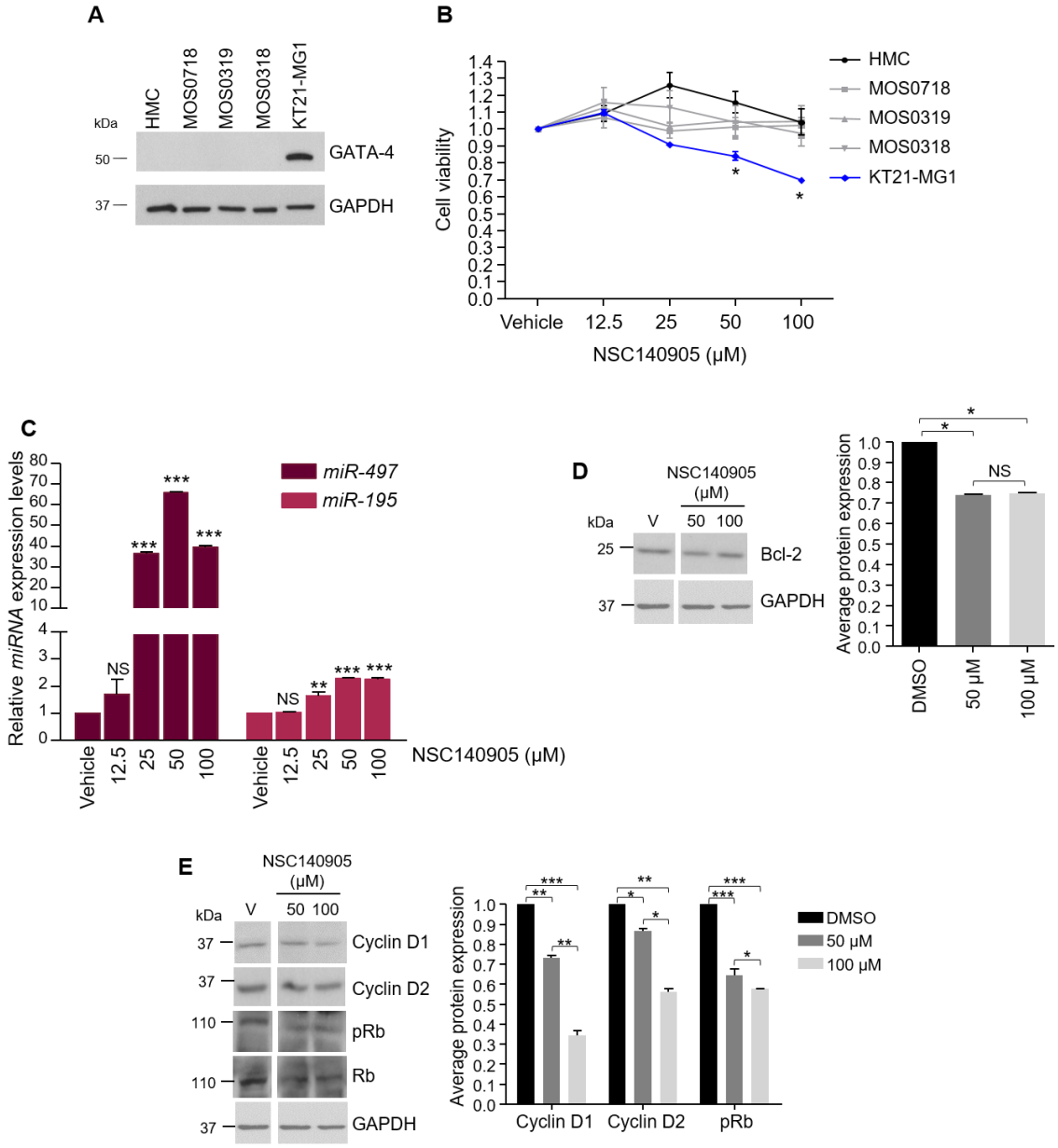


Figure 3.21 Effects of NSC140905 on KT21-MG1 cell viability and miR-497~195 cluster expression. (A) Western blot analysis of GATA-4 expression in HMC, primary Schwann cells (MOS0718, MOS0319, and MOS0318) and KT21-MG1 cell line. (B) Cell viability assessed by ATP assay on HMC, primary Schwann cells (MOS0718, MOS0319, MOSS0318), and KT21-MG1 after treatment with NSC140905 for 24 hours. (C) Decreased transcriptional activity of GATA-4 after administration of NSC140905 was confirmed by following the increase of miR-497 (62.82 and 32.59 fold change at 50 and 100 μM,

respectively) and miR-195 (2.29 and 2.24 fold change at 50 and 100 μM, respectively). Data have been normalised to the average expression in vehicle-treated cells (0.1% DMSO) and shown as mean ± SEM; the expression levels of the snRNA U6 were used as a reference. (D) Western blot panel showing decrease in Bcl-2 after inhibition of GATA-4. (E) Western blot showing decrease in the levels of Cyclin D1 and D2 and in the phosphorylation status of RB. (NS = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$). n = 3.

3.4.2 NSC140905 shows potential to synergize with PD0332991 in decreasing viability of malignant meningioma cells

PD0332991, commercially known as Palbociclib, is a well-tolerated, orally available, highly selective inhibitor of Cyclin D kinases (CDK4/6), with the ability to contrast retinoblastoma protein (RB) phosphorylation in the nanomolar range (Finn *et al.*, 2009). It has been approved by the FDA as a treatment for patients with oestrogen receptor positive (ER+) advanced breast cancer in combination with Letrozole or Fulvestrant (Finn *et al.*, 2015; Finn *et al.*, 2016), and is currently in clinical trial for a number of other malignancies, such as previously treated metastatic breast cancer (Rossi *et al.*, 2019; Turner *et al.*, 2019), gastrointestinal stromal tumours (Toulmonde *et al.*, 2019), and mantle cell lymphoma (Martin *et al.*, 2019), either as a single agent or in combination with different drugs.

Under normal conditions, the cell cycle functions as a tightly regulated and predictable process consisting of distinct phases: G_0 (quiescence), followed by G_1 (pre-DNA synthesis), S (DNA synthesis), G_2 (pre-division), and M (cell

division). Passage from G_1 to S is a key checkpoint in protecting cells from abnormal replication; key in this process is the interaction between the Cyclin-dependent kinases (CDKs) and Cyclin proteins. Hyperphosphorylation of the *RB* gene product, pRB, is mediated in early G_1 by CDK4 and CDK6 interacting with Cyclin D1; once RB is phosphorylated, it becomes inactive, leading to the release of transcription factors that allow progression to the S phase (Lundberg & Weinberg, 1999; Poon, 2016).

Since the malignant meningioma cell line KT21-MG1 displays upregulation of Cyclins belonging to the Cyclin D family (D1, D2, and D3), which are able to interact with and activate CDK4/6 (Hydbring, Malumbres & Sicinski, 2016), I decided to test the efficacy of PD0332991 in inhibiting cell viability.

I hypothesised that the GATA-4 small molecule inhibitor could increase its efficiency in malignant meningioma as both NSC140905 and PD0332991 target proteins involved in the same signalling pathway, *i.e.* cell cycle progression.

3.4.2.1 KT21-MG1 cells show limited sensitivity to the CDK4/6 inhibitor PD0332991

D-family Cyclins need to interact with CDK4/6 in order to activate them and exert their functional roles (Hydbring, Malumbres & Sicinski, 2016).

Since the malignant meningioma cell line KT21-MG1 demonstrates overexpression of Cyclin D1, D2, D3 (as reported above in Fig. 3.1C and D), and of CDK4, compared to both HMC and Ben-Men-1 (5.2 fold change, compared to Ben-Men-1; see Fig. 3.2), I hypothesised that they could display

sensitivity to PD0332991, a selective CDK4/6 inhibitor currently in clinical trial for other malignancies (Finn *et al.*, 2009; Rossi *et al.*, 2019; Turner *et al.*, 2019; Toulmonde *et al.*, 2019; Martin *et al.*, 2019). Therefore, KT21-MG1 cells were treated with increasing concentrations of PD0332991 (1.25 - 2.5 - 5 - 10 μM, plus vehicle - sterile milliQ water - as control) for 24 hours, as previously reported (Finn *et al.*, 2009).

Interestingly, ATP assay, used to assess cell viability (see 2.2.2.1), showed that KT21-MG1 sensitivity to PD0332991 is very limited, demonstrating a significant decrease of cell viability only at the highest concentration tested (10 μM; ~14% decrease, compared to vehicle) (Fig. 3.22).

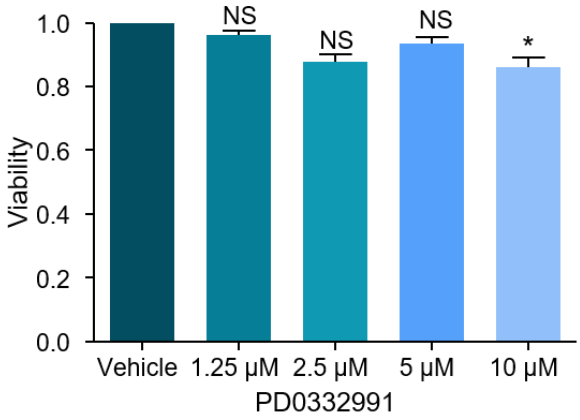


Figure 3.22 KT21-MG1 cells viability following treatment with PD0332991 for 24 hours. A significant decrease in cell viability using an ATP assay was only demonstrated at 10 μM. Data shown have been normalised to the vehicle-treated cells and reported as mean ± SEM. Vehicle = sterile milliQ water. (NS = not significant; * = $p<0.05$). n = 3.

3.4.2.2 Preliminary data show that pre-treatment with NSC140905 enhances PD0332991 effects on cell viability

Since both the inhibitors NSC140905 and PD0332991 have convergent effects on pro-proliferative pathways (the former inhibiting GATA-4 which possibly controls Cyclin D *via* miR-497~195, and the latter blocking the kinase activity of Cyclin D-CDK4 *via* CDK4), I hypothesised that pre-treatment with NSC140905 could increase cellular sensitivity to PD0332991. Therefore, I pre-treated malignant meningioma cells with 50 or 100 μM NSC140905 for 24 hours, prior to administration of PD0332991 before testing viability, as described in section 2.2.2. Combination treatment has only been performed in duplicate.

Although confirmation of the following observations is warranted, from these preliminary data I could observe a significant decrease in viability for all drug combinations tested when compared to controls, except for 50 μM NSC140905 + 5 μM PD0332991, which led to a non-significant decrease in viability (Fig. 3.23A).

As shown in Fig. 3.23B, the highest effect of the combination on KT21-MG1 cells was observed following pre-treatment with 100 μM NSC140905 prior to administration of 10 μM PD0332991, leading to a further 28% reduction in cell viability when compared to single treatment with 10 μM CDK4/6 inhibitor (which only led to a ~14% decrease).

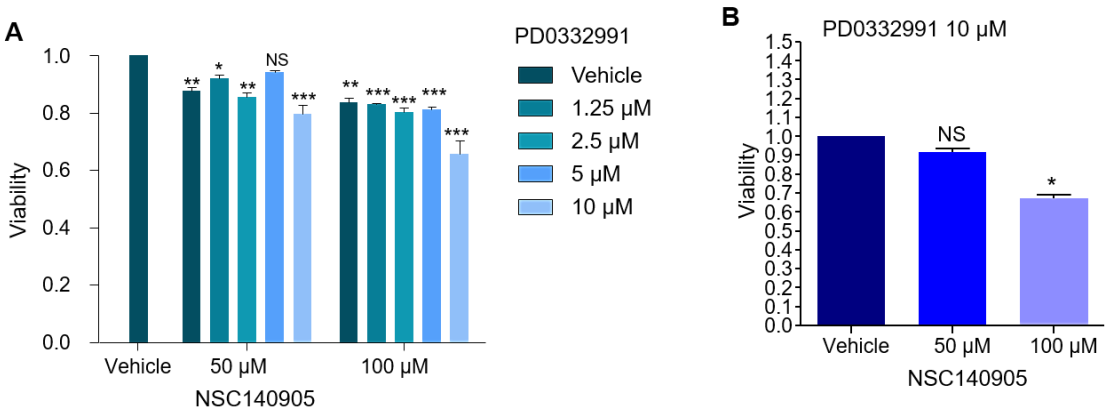


Figure 3.23 Effect of NSC140905 pre-treatment on KT21-MG1 cells. (A) ATP assay revealed significant decreases in cell viability at all the combinations tested, except for 50 µM NSC140905 + 5 µM PD0332991, at which I only observed a 0.04% reduction in viable cells compared to vehicle treated cells (n = 2). Control samples were pre-treated with 0.1% DMSO for 24 hours and then administered sterile milliQ water for 24 hours (respective drug vehicles). (B) Comparison of cell viability following pre-treatment with NSC140905 or single treatment with PD0332991 (n = 2). Data shown have been normalized to the average viability in vehicle-treated (A) or PD0332991 treated (B) cells and reported as mean ± SEM. (NS = not significant; * = $p<0.05$; * = $p<0.01$; *** = $p<0.05$).

3.4.2.3 Investigation of possible synergistic effects between NSC140905 and PD0332991

Since preliminary data showed a decrease in cell viability following pre-treatment with NSC140905, I wanted to understand whether the changes observed could be due to a possible synergistic mechanism of the two drugs tested.

Therefore, cell viability data were entered into CompuSyn (Chou, 2010), a free, widely available software for the calculation of the combination index (CI) in order to understand whether two drugs display an additive effect (CI = 1), synergism (CI < 1) or antagonism (CI > 1) in drug combination experiments. This is a powerful tool to understand whether the combination of two or multiple drugs proves to be more successful than the administration of a single agent, with synergism and antagonism defined as a greater or lesser effects for drugs in combination than the simple additive effect expected from the knowledge of the effects of each drug individually (Foucquier & Guedj, 2015).

Table 3.5 shows the data points entered into the software, whereas Fig. 3.24 shows the normalized isobologram returned by the algorithm.

Even though a high variability was observed, probably due to plating mistakes (*e.g.* inconsistent cell densities, mislabelled treatments) or problems with the stock of drugs used, a CI < 1, suggestive of a synergistic effect, was observed for four combinations: 1.25 μM PD0332991 + 100 μM NSC140905, 2.5 μM PD0332991 + 100 μM, 5 μM PD0332991 + 100 μM and 10 μM PD0332991 + 100 μM (data points 2, 4, 6, and 8, respectively).

Point #	Dose PD0332991	Dose NSC140905	Effect (Mortality)	CI
1	1.25 μM	50 μM	0.08	2.9
2	1.25 μM	100 μM	0.17	0.92
3	2.5 μM	50 μM	0.12	1.28
4	2.5 μM	100 μM	0.2	0.64
5	5 μM	50 μM	0.04	18.34
6	5 μM	100 μM	0.19	0.83
7	10 μM	50 μM	0.16	1.06
8	10 μM	100 μM	0.34	0.19

Table 3.5 Data entered in CompuSyn. CompuSyn allows the entry of drug effect as values between 0 and 1 (0 and 1 excluded); therefore, I calculated the mortality as 1-(cell viability) observed in the ATP assay. Mortality observed following single treatment with either drugs has been entered as a reference. CI < 1 = synergism; CI = 1 = additive effect; CI > 1 = antagonism.

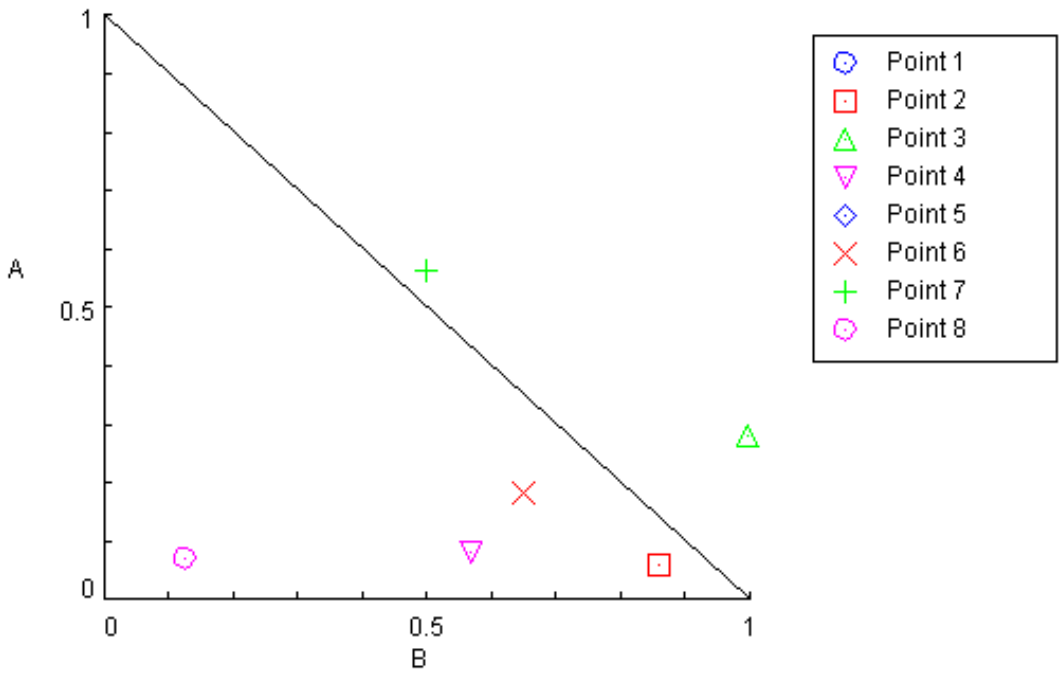


Figure 3.24 Normalised isobologram for NSC140905 + PD0332991 combination. Data points on the lower left of the hypotenuse in the isobologram symbolize drugs with a synergistic effect (defined as greater than expected additive effect, points # 2, 4, 6, and 8); points on the upper-right of the hypotenuse represent antagonistic combinations (smaller than expected additive effect), whereas points on the hypotenuse represent additive effect (the combined effect predicted by the mass-action law principle in absence of synergism or antagonism). Data points 1 and 5 are not present in the normalized isobologram as they fall outside of its range.

3.4.2.4 Investigation of the effects caused by treatment with synergistic doses of PD0332991 and NSC140905

Since CompuSyn returned a CI of borderline significance for the combinations 1.25 μM PD0332991 + 100 μM NSC140905 and 5 μM PD0332991 + 100 μM (CI = 0.92 and 0.83, respectively), I decided to focus the following experiments on the combinations with a lower CI, *i.e.* 2.5 μM PD0332991 + 100 μM and 10 μM PD0332991 + 100 μM (CI = 0.64 and 0.19, respectively, see Table 3.5).

Therefore, cells were treated according to these schedules and cell lysates were collected to investigate the effects of the drug combinations at the protein level. This experiment was performed in duplicate, therefore at least one more biological repeat is necessary to confirm these findings.

Preliminary data show that, at both concentrations, PD0332991 inhibits CDK4 and CDK6 activity, as indirectly demonstrated by lack of detection of phospho-RB *via* Western blotting (Fig. 3.25A and B, respectively). Interestingly, treatment with 2.5 μM PD0332991 led to a 4% decrease in the expression levels of Cyclin D1, whereas Cyclin D2 showed a trend to increase compared to vehicle treated cells (~17% increase). When cells were pre-treated with 100 μM NSC140905, the expression levels of Cyclin D1 decreased (~33%), whereas Cyclin D2 showed a trend to decrease (~34% decrease, respectively) compared to single treatment with the CDK4/6 inhibitor (Fig. 3.25A).

When cells were treated with the highest concentration of PD0332991, a significant decrease in both Cyclin D1 and D2 (59% and 19% decrease compared to vehicle treated cells) was observed, further accentuated by pre-

treatment with 100 μM NSC140905 (~33% and 29% decrease, respectively, compared to single treatment with PD0332991) (Fig. 3.25B).

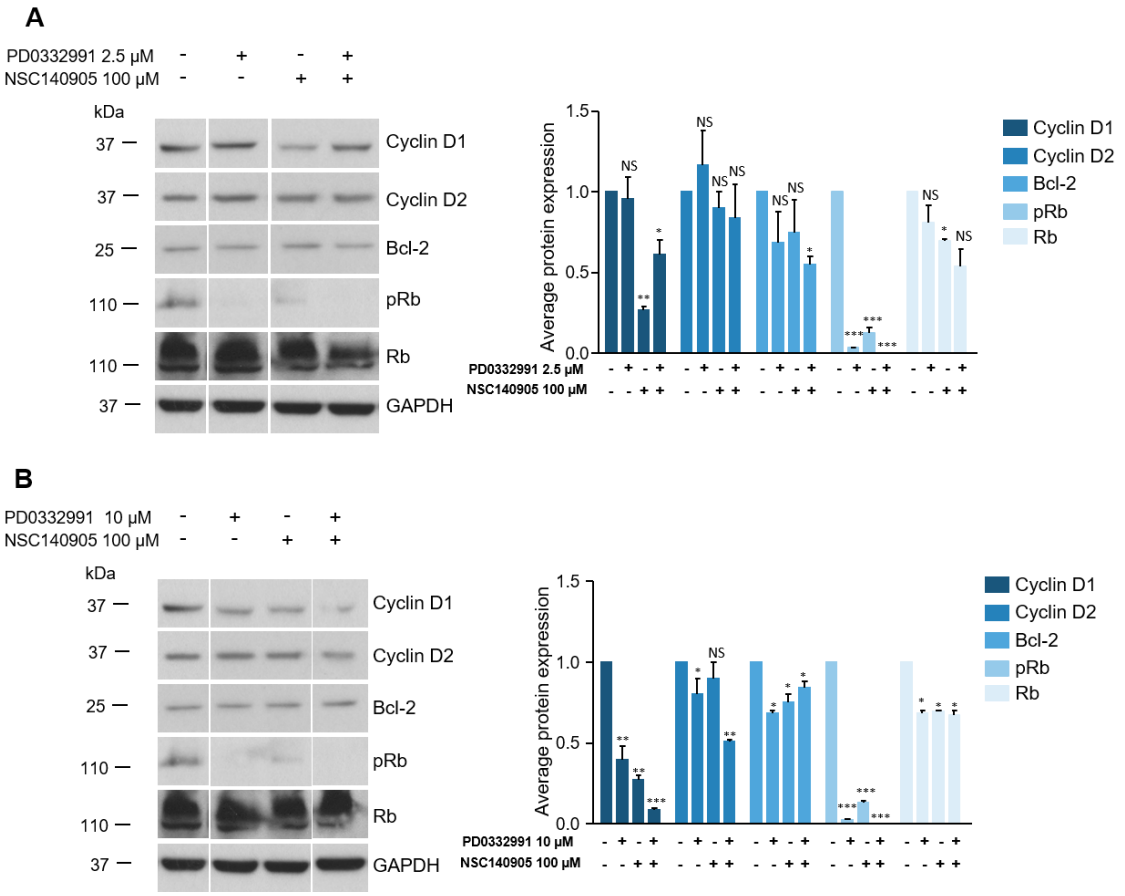


Figure 3.25 Representative Western blot panels of the effects of treatment with the synergistic combinations of PD0332991 and NSC140905. (A) Effects of pre-treatment with 100 μM NSC140905 followed by treatment with 2.5 μM PD0332991 on KT21-MG1 cells. (B) Effects of pre-treatment with 100 μM NSC140905 followed by treatment with 10 μM PD0332991. GAPDH was used as a loading control. n = 2.

3.4.3 Discussion

GATA-4 is a transcription factor significantly upregulated in malignant meningioma cells and tissues, displaying a good diagnostic value when comparing WHO I samples with WHO II-III tumours, as shown in section 3.3 by performing ROC curve analysis. Therefore, it could be a potential novel therapeutic target for higher-grade meningiomas, whose clinical management is particularly challenging (Mawrin, Chung & Preusser, 2015).

Transcription factors have long been deemed as 'undruggable' targets but the increasing knowledge available about their precise roles and the advancement in techniques to study their interaction with co-factors and DNA-binding sites have opened new possibilities to modulate their activity (Lambert *et al.*, 2018).

Although to date there are no approved drugs to target GATA-4, a study in 2011 identified four small molecular weight chemicals with lead-like properties able to bind to the DNA-binding domain of GATA-4, thus reducing its transcriptional activity (El-Hachem & Nemer, 2011).

One of those four molecule inhibitors, NSC140905, a derivative of succinic acid, proved to be more efficient than the others in inhibiting GATA-4 at the lowest dose tested by the authors (50 μM). Efficiency of the compounds was tested on a cell line (HeLa) that does not naturally express GATA-4, having to overexpress the protein and then treat the cells with the inhibitor (El-Hachem & Nemer, 2011).

Therefore, I wanted to investigate the effect of this compound on the malignant meningioma cell line KT21-MG1, which naturally expresses high levels of GATA-4.

Indeed, KT21-MG1 cells proved to be sensitive to inhibition of GATA-4 by NSC140905, with results at the highest doses tested (50 and 100 μM) that were in line with those observed in the previous section following lentiviral-mediated knockdown of the protein (section 3.3, Fig. 3.16).

Following treatment with the small molecule compound, a decrease in cell viability, decreased levels of the GATA-4 target Bcl-2, and increased levels of the miR-497~195 cluster were observed, which led to a decrease of Cyclin D1 and D2 expression and to a reduction in the phosphorylation status of the Retinoblastoma protein (Fig. 3.21C, D and E).

As in the previous section, I assessed cell viability by ATP assay, which measure the metabolic activity of the cells, and can therefore be biased by the presence of quiescent cells, which are metabolically inactive.

It could be interesting to understand whether GATA-4 inhibition leads to more general effects, like cell cycle inhibition, and, if so, in which phase. This investigation could lead us to understand molecular mechanisms that could contrast the effects of NSC140905 (*i.e.* expression of the transcription factor E2F1 or PIN1) (Farra *et al.*, 2015), eventually promoting pharmaco-resistance.

Interestingly, NSC140905 proved not to be active when tested on normal human meningeal cells (HMC), which do not express GATA-4 at measurable levels, thus suggesting that the molecule is highly selective for GATA-4 and that it does not interfere with other transcription factors involved in the regulation of cell proliferation and cell death.

These results were supported also by testing the GATA-4 inhibitor in a panel of three different primary Schwann cells (MOS, Fig. 3.21B).

Similarly to HMC, Schwann cells do not express GATA-4 and their viability was not affected by treatment with NSC140905 either, confirming lack of activity on healthy primary cells (Fig. 3.21A and B); however, it should be noted that I only performed the ATP assay after 24 hours from NSC140905 administration, therefore it would be essential to monitor cell viability at longer time points before extensive evaluation of this drug in any preclinical model.

Furthermore, it has been shown that GATA-4 is an important survival factor for differentiated, postnatal cardiomyocytes and that GATA-4 depletion is an early event in the cardiotoxic effect of the antitumor drug Doxorubicin (Aries *et al.*, 2004).

Although GATA-4 has been shown to be expressed in neurons and glia of normal murine and human embryonic and adult CNS, its silencing in normal astrocytes did not alter their growth properties (Agnihotri *et al.*, 2009). However, GATA-4 depletion by either deletion or epigenetic silencing is implicated in many malignancies, such as colorectal, oesophageal, gastric, lung, ovarian and HPV-driven oropharyngeal cancer, glioblastoma multiforme, and diffuse large B-cell lymphoma (Lin *et al.*, 2000; Akiyama *et al.*, 2003; Guo *et al.*, 2004; Derks *et al.*, 2006; Guo *et al.*, 2006; Wakana *et al.*, 2006; Pike *et al.*, 2008; Hellebrekers *et al.*, 2009; Agnihotri *et al.*, 2011; Kostareli *et al.*, 2013).

These observations warrant caution in the formulation of potential drugs inhibiting GATA-4 and careful pre-clinical studies to determine any possible toxicity.

Since increased levels of Cyclin D1, D2, D3, and of CDK4 in the malignant meningioma KT21-MG1 cell line were observed, I decided to investigate the efficacy of PD0332991, an orally available selective inhibitor of CDK4/6,

currently in clinical trial for metastatic breast cancer (Rossi *et al.*, 2019; Turner *et al.*, 2019), gastrointestinal stromal tumours (Toulmonde *et al.*, 2019), and mantle cell lymphoma (Martin *et al.*, 2019), either as a single agent or in combination.

KT21-MG1 cells proved to be resistant to treatment with PD0332991, with a significant decrease in cell viability being observed only at the highest dose tested (10 μM, Fig. 3.22); this could be due to the high levels of Cyclin E1 (see Fig. 3.1C), which has been shown to be involved in resistance to the CDK4/6 inhibitor (Guarducci *et al.*, 2018; Min *et al.*, 2018).

Therefore, I investigated whether the combination of NSC140905, which led to a reduction of the expression levels of Cyclin D1, D2, and possibly D3, and E1, with PD0332991 could have a synergistic effect in decreasing the proliferative capacity of KT21-MG1 cells.

The advantage of using combination therapies is the possibility to decrease the doses of both drugs used, thus minimizing toxicity and preventing the development of drug resistance (Bayat Mokhtari *et al.*, 2017).

Combination experiments have only been performed in duplicate, and a high variability in results was observed (*e.g.* high variability in the ATP assay, and thus in the calculation of combination index, Fig. 3.23 and Table 3.5, as well as in the Western blot panels, Fig. 3.25; this was probably due to plating mistakes, *e.g.* inconsistent cell densities, or to problems with the stock of drugs used); therefore, data presented here are only exploratory and warrant further confirmation.

From the preliminary data available, I observed that, although pre-treatment with 50 μM NSC140905 led to a significant decrease in cell viability compared to cells treated with PD0332991 as a single agent (Fig. 3.23A), calculation of the combination index for the drug combination revealed that only the highest dose of NSC140905 (100 μM) led to a synergic effect with PD0332991 when used at 2.5 and 10 μM (Table 3.5).

Interestingly, at both concentrations of PD0332991 tested, I was not able to detect Retinoblastoma protein (pRB) phosphorylation *via* Western blot analysis, suggesting indirectly that the drug is efficiently inhibiting CDK4/6 activity (Fig. 3.25A and B). However, at the lowest dose of PD0332991 (2.5 μM) a trend to increase of Cyclin D2 was observed (Fig. 3.25A). At least one further biological repeat is warranted to confirm this observation, which could be due to a compensatory mechanism to overcome the inhibition of CDK4/6, as it has been shown that Cyclin D2 is upregulated by cells in stressful conditions, such as biomechanical stress and serum starvation (Meyyappan *et al.*, 1998; Angelis *et al.*, 2008). Moreover, D-type Cyclins have been shown to be able to regulate transcription of target genes in a CDK4/6 and pRB independent manner (Skapek *et al.*, 1996; Fu *et al.*, 2005; Du, Tong & Ye, 2013), therefore upregulation of Cyclin D2 could enable the continuation of cell cycle despite inhibition of CDK4/6.

As preliminary experiments show that the combination of NSC140905 with PD0332991 did not allow me to decrease the dose of the small molecule GATA-4 inhibitor, we should look at other drug combinations that might lead to a reduction of NSC140905 needed to decrease cell viability in order to minimize side effects.

In this section, for the calculation of the combination index I used the CompuSyn software, which relies on a dose-effect-based approach that leads to the calculation of an isobologram, a graph indicating the equipotent combinations of various doses of two drugs. This type of assessment can be used to illustrate additive effect, synergism or antagonism of the two compounds. As the concentrations of the two drugs tested were not in a constant ratio, the programme returned the normalised isobologram, which displays the normalised dose of drug 1 (in this case PD0332991) and drug 2 (NSC140905) on the *x*- and *y*- axes, respectively (Fig. 3.24) (Chou, 2010).

The advantage of using this program is that it allows a reduction in the number of data points required to yield useful information on the relationship between dose and effect (Chou, 2011), whereas other approaches based on the Loewe Additivity rely on a larger amount of data and can rapidly become expensive as well as experimentally and computationally demanding (Lehar *et al.*, 2007).

4 Discussion

4.1 Introduction

Cyclin D1 has been suggested as a molecular biomarker for meningioma due to its overexpression in tumour tissues when compared to normal meninges, and its levels correlate with the proliferative activity of the tumour, which has prognostic significance (Alama *et al.*, 2007; Milenkovic *et al.*, 2008). However, its detection has been carried out *via* techniques that require tissue availability, such as immunohistochemical staining or Western blot analysis of tumour lysates.

The aim of this project was to elucidate the mechanisms involved in the regulation of Cyclin D1 expression levels in meningioma at the post-transcriptional levels, by focusing on the study of microRNAs in WHO I, II and III meningioma tissues. I also examined matched blood samples, in order to identify new molecular biomarkers, which could be detected in peripheral blood, a much less invasive method of sample collection from patients.

The importance of miRNAs in oncogenesis has been recognised, with dysregulation of miRNA expression shown to play a key role in cancer development through various mechanisms including deletions, amplifications, epigenetic silencing or mutations in miRNA *loci*, and dysregulation of transcription factors that target specific miRNAs (Kosaka, Iguchi & Ochiya, 2010).

A number of studies recently focussed on miRNA networks in meningioma (Kliese *et al.*, 2013; Zhi *et al.*, 2013; Wang *et al.*, 2015; Ludwig *et al.*, 2015; Zhi

et al., 2016; El-Gewely *et al.*, 2016), showing that they have potential application as diagnostic and prognostic tools in clinical practice.

The advantage of studying miRNAs is that they can be detected in peripheral blood either as free circulating RNAs or as cargo of extracellular vesicles, including exosomes (Zhi *et al.*, 2016). Blood-based biomarkers have the important benefit of being minimally invasive compared to tissue biopsy, high-throughput and affordable (Ma, Jiang & Kang, 2012; Zhi *et al.*, 2016). Moreover, they allow easy and non-invasive repeat sampling from the same patient.

However, studies so far have investigated changes in miRNA levels extracted from whole serum. This miRNA fraction is not only composed of miRNA secreted from tumour cells, but also from nucleic acids released into the bloodstream following cell death and lyses, thus including miRNAs that are not necessarily associated with cancer (Ma, Jiang & Kang, 2012). On the other hand, production and secretion of EVs is increased in cancer cells (Bebelman *et al.*, 2018), and it has been shown that blood of cancer patients is twice as rich in exosomes as the blood of healthy individuals (Kalluri, 2016). These observations highlight exosomes' potential to be used as liquid biopsies to support cancer diagnosis and/or prognosis.

For the first time, the research described in this study shows that the downregulation of the miR-497~195 cluster, which regulates Cyclin D1, in malignant meningioma is due to the upregulation of the transcription factor GATA-4, which I speculate could represent a potential novel tissue-specific marker for malignant meningioma as well as a new therapeutic target. Moreover, my findings show that miR-497 could be a potential novel circulating biomarker for higher-grade meningioma (WHO II-III).

The following sections of this chapter will discuss the results obtained during the course of this project and their relevance in relation to meningioma, as well as suggest future research to build upon these findings.

4.2 Discovery of novel biomarkers for meningioma

Despite the genetic landscape of meningioma having been described, meningioma classification is still based on histopathological characterization of tumours, a method that relies on the availability of tumour tissue, which can be challenging to obtain depending on tumour location, is subjected to inter-observer variability, and has been questioned due to the little prognostic effect of the histological criteria of higher-grade meningioma (Rogers *et al.*, 2016; Vaubel *et al.*, 2016; Baumgarten *et al.*, 2016). All these shortcomings highlight the need to find novel molecular biomarkers for meningioma grading.

In this project, I identified microRNAs involved in the regulation of Cyclin D1 *via* a search on open-access databases; the candidates have been validated by qPCR on 125 meningioma tissue samples across all WHO grades, 39 matched meningioma patients' serum samples, 39 PDMN cells (WHO I and II) and two meningioma cell lines, the benign Ben-Men-1 and the malignant KT21-MG1. Moreover, I investigated the mechanism involved in the regulation of the candidate miRNAs, and found a novel potential tissue-specific biomarker and therapeutic target for malignant meningioma.

Unfortunately the investigations outlined in this thesis have been limited by some difficulties encountered with our *in vitro* meningioma model. Although some WHO II primary cell samples could be derived, these cells grew very

slowly and for a very limited period of time (~2-4 passages). Therefore, I chose to collect only RNA from WHO II PDMN cells, as I could obtain sufficient amount of RNA from such limited amount of cells.

Additionally, our research group have, thus far, been unsuccessful in amplifying WHO III PDMN cells; therefore all investigations in malignant meningioma have been performed using the KT21-MG1 cell line for this study.

Another shortcoming is that I stratified meningioma samples based on the WHO histological characterization, which is the current gold standard for grading, due to the limited sample size available, especially regarding WHO II and III samples. If more tissues could be sourced, analysis of the data based on genetic subgrouping of the samples could highlight whether samples sharing the same mutational pattern display the same molecular signature with regards to the miR-497~195 cluster and GATA-4.

Methylation analysis on all, or part, of the samples used in this study could also be useful to investigate whether samples with higher levels of GATA-4 and lower levels of miR-497 and -195 cluster in the malignant class; this would allow linking of molecular characteristic of the tumours with their biological behaviour.

4.2.1 miR-497 and -195 as novel potential biomarkers for meningioma

I have shown that miR-497 and -195 could be novel tissue-specific biomarkers for higher-grade meningioma (WHO II-III) as both candidates showed a good diagnostic value when considered alone and as part of a 2-miRNA signature.

This observation was important as it is essential to evaluate more than one parameter to draw reliable conclusions.

When the expression of both miRNAs in exosomes isolated from patients' serum samples was investigated, only miR-497 proved to have a good diagnostic value, whereas the ROC curve profile of miR-195 was not indicative of good sensitivity and specificity, as the curve almost intersected the line of identity in more than one point.

This could be due to the low number of samples available (24 WHO I serum samples, 10 WHO II, and 5 III samples). This limitation led me to evaluate differences between benign meningioma and 'higher-grade' samples, grouping WHO II and III patients together.

One future direction of this pilot analysis will be to further validate my preliminary observations by analysing more blood samples, ungrouping WHO II patients from WHO III, and monitoring the levels of the two candidates at all degrees of malignancy.

Moreover, I performed a screening in exosomes only on blood samples collected prior to surgery. However, it would be interesting to analyse circulating miRNA signatures after surgery, following adjuvant treatment, and in the event of tumour recurrence to identify changes in circulating miR-497 and -195, if there are any, in order to understand if the candidates have potential to be used as prognostic biomarkers for clinical outcome.

4.2.2 GATA-4 as a potential tissue-specific biomarker for malignant meningioma

While investigating the potential mechanisms involved in miR-497~195 cluster regulation, I observed that the expression levels of the transcription factor GATA-4, previously suggested as a potential regulator of the miR-15 family (Yu *et al.*, 2013), negatively correlated with the expression levels of miR-497 and -195 in malignant meningioma samples.

When comparing atypical and benign meningioma samples, I observed a trend to increase of GATA-4 in WHO II tissues, but the difference was not of statistical significance, whereas both miR-497 and -195 are significantly downregulated in WHO II samples when compared to WHO I.

This could be due to the shortcomings of the applied histopathological criteria. Brain invasion has been introduced as a histological criterion that can alone suffice for diagnosing an atypical meningioma (Louis *et al.*, 2016), even though these tumours might show a clinical course similar to WHO I.

A study published in 2017 suggested that meningioma grading based on their genome-wide methylation pattern was more useful than the current WHO grading criteria in classifying meningioma in different malignancy groups, and proved superior in the prediction of progression-free survival (Sahm *et al.*, 2017).

However, the samples included in this study have been stratified according to the current WHO classification criteria; therefore, it could be that some tumours fall in one category even though they display a more or less aggressive behaviour. Re-grading of the tumour tissues used in this study exploiting an

integration of the DNA-methylation system with the current WHO criteria should enable the validation of the results outlined in this thesis.

Nonetheless, I speculate that GATA-4 could be a novel molecular biomarker for malignant meningioma, as it proved to have a good diagnostic value in ROC curve analysis performed comparing low-grade (WHO I samples) with high-grade meningioma (WHO II and III samples).

4.3 GATA-4 controls the expression of the miR-497~195 cluster in meningioma

As it was observed that GATA-4 expression levels negatively correlated with those of the miR-497~195 cluster, and this transcription factor is predicted to have binding sites on the miR-497~195 cluster promoter (Fishilevich *et al.*, 2017), I investigated the effect of lentiviral-mediated *GATA-4* transduction or RNA interference in the two meningioma cell lines available.

Indeed, overexpression of *GATA-4* in Ben-Men-1 led to a downregulation of the miR-497~195 cluster and in turn to an increase in Cyclin D1, D2, D3, and E1 expression levels, an increment in the phosphorylation status of RB, and increased cell viability.

Conversely, lentiviral-mediated *GATA-4* knockdown in KT21-MG1 cells led to an increase in miR-497~195 cluster expression levels, resulting in decreased cell viability compared with scramble control.

Since the promoter/enhancer region of the miRNA cluster is not known, but can only be predicted *via* bioinformatics analysis based on its genomic location, I

could not prove a direct binding of GATA-4 on miR-497~195 cluster promoter. Therefore, I cannot confidently say whether the effects observed following lentiviral-mediated overexpression or RNA interference of *GATA-4* are directly due to increased/decreased binding of GATA-4 to the promoter of the miRNA cluster or are an indirect consequence of other mechanisms.

The 3' UTR of *GATA-4* is predicted to harbour one binding site for miR-15 family members; hence I decided to evaluate the effect of miR-195 overexpression in KT21-MG1 cells, which also allowed me to confirm miR-195 involvement in the regulation of Cyclin D1, D2, D3, and E1 expression levels in meningioma.

Indeed, lentiviral-mediated transduction of miR-195 in KT21-MG1 cells led to a decrease of GATA-4 expression, both at the transcriptional and protein levels.

As a feedback regulatory loop is often present between miRNAs and the proteins coded by its targeted mRNAs (Quan *et al.*, 2019; Yang *et al.*, 2019; Hou *et al.*, 2019; He *et al.*, 2019), this observation strengthens the hypothesis that GATA-4 is directly involved in the regulation of the miR-497~195 cluster expression.

This observation could be further supported by future experiments performing reporter assays in order to demonstrate without doubt binding of miR-195 on the 3' UTR of *GATA-4* mRNA.

Nonetheless, my experiments show a relationship between the miR-497~195 cluster and the transcription factor GATA-4 in meningioma.

4.4 GATA-4 as a potential therapeutic target for malignant meningioma

I observed that GATA-4 expression was higher in malignant meningioma samples, representing a potential novel therapeutic target for malignant meningioma, whose clinical management is especially challenging (Mawrin, Chung & Preusser, 2015).

Unfortunately, there are no approved drugs available to target GATA-4 activity, but a screening performed in 2011 led to the identification of some small molecular weight compounds able to bind to the C-terminal zinc finger of the protein, thus inhibiting its ability to bind to the DNA (El-Hachem & Nemer, 2011).

I tested one of those molecules, NSC140905, in the malignant meningioma cell line KT21-MG1, and found that inhibition of GATA-4 led to upregulation of the miR-497~195 cluster and reduced cell viability, similarly to the effects observed after lentiviral-mediated RNA interference of *GATA-4*.

GATA-4 has been shown to be essential for cardiomyocytes survival, and its depletion is linked to cardiotoxicity, *e.g.* following administration of Doxorubicin (Aries *et al.*, 2004).

Moreover, GATA-4 downregulation is implicated in many different tumours (Lin *et al.*, 2000; Akiyama *et al.*, 2003; Guo *et al.*, 2004; Derks *et al.*, 2006; Guo *et al.*, 2006; Wakana *et al.*, 2006; Pike *et al.*, 2008; Hellebrekers *et al.*, 2009; Agnihotri *et al.*, 2011; Kostareli *et al.*, 2013); these evidences warrant caution in the formulation of potential GATA-4 inhibitors and careful pre-clinical studies to determine any possible undesired effect. However, I could not find an effect of NSC140905 on neither normal meningeal cells nor primary Schwann cells.

Ways to overcome potential cardiac toxicity and oncogenic functions of GATA-4 inhibitors would be to functionalise them in order to direct them only to the sites in which they are needed, avoiding systemic delivery of the drug.

Moreover, if GATA-4 inhibitor drugs were formulated, it would be important to evaluate their effects as part of a combination therapy, which would allow reduction of the doses needed to obtain a positive effect on tumour growth, thus limiting the development of side effects.

4.5 Conclusion

The aim of this project was to elucidate the post transcriptional mechanisms involved in the regulation of Cyclin D1 expression levels in meningioma, by focusing on the study of microRNAs in WHO I, II and III meningioma tissues and matched blood samples, in order to identify new molecular biomarkers which could be detected in peripheral blood.

To my knowledge, this is the most comprehensive study of meningioma-associated miRNAs expression levels and functions, covering validation in 125 meningioma tissues of all grades, including 12 malignant meningioma samples, 39 matched patients' serum samples and 39 benign and atypical patient-derived meningioma primary cell samples. In addition, analysis of the candidate miRNAs enabled discovery not only of two tissue-specific (2-miRNA signature, and GATA-4 for high-grade meningioma tumours) and circulating (miR-497) meningioma biomarkers, but also a novel potential therapeutic target for the treatment of high-grade meningiomas.

This body of work provides the basis of future projects to further validate my results in bigger cohorts of meningioma patients and evidence for potential usefulness of GATA-4 inhibitors in malignant meningioma.

4.6 Future work

Some future experiments are warranted to further confirm the findings presented in this thesis.

Collection of a larger amount of serum samples would allow validation of miR-497, and possibly -195, as circulating biomarkers; moreover, access to serial samples from the same patient before and after surgery, at time of treatment, and at time of recurrence, would allow investigation of miR-497 and -195 prognostic significance, and whether they could be used as biomarkers for clinical outcome.

Investigation of the methylation profile of the samples used in this study would allow to establish whether samples clustering in a specific class share the same molecular characteristics regarding the miR-497~195 cluster and GATA-4 expression; furthermore, collection of a larger amount of samples would also permit stratification based on the genetic subgroup of the tumours, to understand whether a certain mutational profile (*e.g. NF2, TRAF7, TRAF7/AKT1, TRAF7/KLF4*) is linked to a different biological behaviour.

Moreover, it would be interesting to understand whether re-introduction of miR-497 and -195, either alone or in combination, into the malignant KT21-MG1 cell line affects *in vivo* tumorigenesis in meningioma xenograft models.

Further studies are warranted on GATA-4 as well; since I have observed a significant upregulation in malignant compared to benign meningioma, a retrospective study of GATA-4 expression on archival tumour tissues would allow us to correlate GATA-4 expression to disease recurrence and survival of patients, to understand whether this factor could have prognostic significance.

Similarly to what has been suggested for miR-497 and -195, it would be interesting to study whether GATA-4 downregulation can affect *in vivo* tumorigenesis in meningioma xenograft models to further highlight its potential as a therapeutic target for high-grade meningioma.

Additionally, I have shown that interfering with *GATA-4* expression leads to changes on the miR-497~195 cluster and on Cyclin D1, D2, D3, and E1 levels, suggesting this latter event to be a consequence of altered miRNA expression. To confirm this hypothesis, a double infection should be carried out in KT21-MG1 cells to understand whether concomitant *GATA-4* and miR-497~195 cluster knockdown rescues the effects observed on Cyclins levels following *GATA-4* knockdown alone. If this was the case, then we could more confidently suggest the involvement of the miR-497~195 cluster in this event.

More extensive evaluations are warranted for the small molecule inhibitor NSC140905 in order to establish its effects on cell viability, proliferation and death of malignant meningioma and explore its potential toxic effects on normal cells (*e.g.* neurons, astrocyte, cardiomyocytes). These investigations could eventually lead to the formulation of a GATA-4 inhibitor drug.

References

Aavikko, M., Li, S. P., Saarinen, S., Alhopuro, P., Kaasinen, E., Morgunova, E., Li, Y., Vesanen, K., Smith, M. J., Evans, D. G., Poyhonen, M., Kiuru, A., Auvinen, A., Aaltonen, L. A., Taipale, J. & Vahteristo, P. (2012) 'Loss of SUFU function in familial multiple meningioma'. *Am J Hum Genet*, 91 (3), pp. 520-526.

Abedalthagafi, M., Bi, W. L., Aizer, A. A., Merrill, P. H., Brewster, R., Agarwalla, P. K., Listewnik, M. L., Dias-Santagata, D., Thorner, A. R., Van Hummelen, P., Brastianos, P. K., Reardon, D. A., Wen, P. Y., Al-Mefty, O., Ramkissoon, S. H., Folkerth, R. D., Ligon, K. L., Ligon, A. H., Alexander, B. M., Dunn, I. F., Beroukhim, R. & Santagata, S. (2016) 'Oncogenic PI3K mutations are as common as AKT1 and SMO mutations in meningioma'. *Neuro Oncol*, 18 (5), pp. 649-655.

Abedalthagafi, M. S., Merrill, P. H., Bi, W. L., Jones, R. T., Listewnik, M. L., Ramkissoon, S. H., Thorner, A. R., Dunn, I. F., Beroukhim, R., Alexander, B. M., Brastianos, P. K., Francis, J. M., Folkerth, R. D., Ligon, K. L., Van Hummelen, P., Ligon, A. H. & Santagata, S. (2014) 'Angiomatous meningiomas have a distinct genetic profile with multiple chromosomal polysomies including polysomy of chromosome 5'. *Oncotarget*, 5 (21), pp. 10596-10606.

Adel Fahmideh, M., Schwartzbaum, J., Frumento, P. & Feychting, M. (2014) 'Association between DNA repair gene polymorphisms and risk of glioma: a systematic review and meta-analysis'. *Neuro Oncol*, 16 (6), pp. 807-814.

Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. (2015) 'Predicting effective microRNA target sites in mammalian mRNAs'. *Elife*, 4

Ageberg, M. & Lindmark, A. (2003) 'Characterisation of the biosynthesis and processing of the neutrophil granule membrane protein CD63 in myeloid cells'. *Clin Lab Haematol*, 25 (5), pp. 297-306.

Agnihotri, S., Wolf, A., Munoz, D. M., Smith, C. J., Gajadhar, A., Restrepo, A., Clarke, I. D., Fuller, G. N., Kesari, S., Dirks, P. B., McGlade, C. J., Stanford, W. L., Aldape, K., Mischel, P. S., Hawkins, C. & Guha, A. (2011) 'A GATA4-regulated tumor suppressor network represses formation of malignant human astrocytomas'. *J Exp Med*, 208 (4), pp. 689-702.

Agnihotri, S., Wolf, A., Picard, D., Hawkins, C. & Guha, A. (2009) 'GATA4 is a regulator of astrocyte cell proliferation and apoptosis in the human and murine central nervous system'. *Oncogene*, 28 (34), pp. 3033-3046.

Aizer, A. A., Abedalthagafi, M., Bi, W. L., Horvath, M. C., Arvold, N. D., Al-Mefty, O., Lee, E. Q., Nayak, L., Rinne, M. L., Norden, A. D., Reardon, D. A., Wen, P. Y., Ligon, K. L., Ligon, A. H., Beroukhim, R., Dunn, I. F., Santagata, S. & Alexander, B. M. (2016) 'A prognostic cytogenetic scoring system to guide the adjuvant management of patients with atypical meningioma'. *Neuro Oncol*, 18 (2), pp. 269-274.

Akers, J. C., Ramakrishnan, V., Kim, R., Skog, J., Nakano, I., Pingle, S., Kalinina, J., Hua, W., Kesari, S., Mao, Y., Breakefield, X. O., Hochberg, F. H.,

Van Meir, E. G., Carter, B. S. & Chen, C. C. (2013) 'MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development'. *PLoS One*, 8 (10), pp. e78115.

Akiyama, Y., Watkins, N., Suzuki, H., Jair, K. W., van Engeland, M., Esteller, M., Sakai, H., Ren, C. Y., Yuasa, Y., Herman, J. G. & Baylin, S. B. (2003) 'GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer'. *Mol Cell Biol*, 23 (23), pp. 8429-8439.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A. & Rak, J. (2008) 'Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells'. *Nat Cell Biol*, 10 (5), pp. 619-624.

Alama, A., Barbieri, F., Spaziante, R., Bruzzo, C., Dadati, P., Dorcaratto, A. & Ravetti, J. L. (2007) 'Significance of cyclin D1 expression in meningiomas: a preliminary study'. *J Clin Neurosci*, 14 (4), pp. 355-358.

Alexander, B. M. & Cloughesy, T. F. (2017) 'Adult Glioblastoma'. *J Clin Oncol*, 35 (21), pp. 2402-2409.

Alqahtani, A., Ayesh, H. S. K. & Halawani, H. (2019) 'PIK3CA Gene Mutations in Solid Malignancies: Association with Clinicopathological Parameters and Prognosis'. *Cancers (Basel)*, 12 (1),

Ammoun, S. & Hanemann, C. O. (2011) 'Emerging therapeutic targets in schwannomas and other merlin-deficient tumors'. *Nat Rev Neurol*, 7 (7), pp. 392-399.

An, O., Dall'Olio, G. M., Mourikis, T. P. & Ciccarelli, F. D. (2016) 'NCG 5.0: updates of a manually curated repository of cancer genes and associated properties from cancer mutational screenings'. *Nucleic Acids Res*, 44 (D1), pp. D992-999.

Angelis, E., Garcia, A., Chan, S. S., Schenke-Layland, K., Ren, S., Goodfellow, S. J., Jordan, M. C., Roos, K. P., White, R. J. & MacLellan, W. R. (2008) 'A cyclin D2-Rb pathway regulates cardiac myocyte size and RNA polymerase III after biomechanical stress in adult myocardium'. *Circ Res*, 102 (10), pp. 1222-1229.

Antinheimo, J., Haapasalo, H., Haltia, M., Tatagiba, M., Thomas, S., Brandis, A., Sainio, M., Carpen, O., Samii, M. & Jaaskelainen, J. (1997) 'Proliferation potential and histological features in neurofibromatosis 2-associated and sporadic meningiomas'. *J Neurosurg*, 87 (4), pp. 610-614.

Anttonen, M., Pihlajoki, M., Andersson, N., Georges, A., L'Hote, D., Vattulainen, S., Farkkila, A., Unkila-Kallio, L., Veitia, R. A. & Heikinheimo, M. (2014) 'FOXL2, GATA4, and SMAD3 co-operatively modulate gene expression, cell viability and apoptosis in ovarian granulosa cell tumor cells'. *PLoS One*, 9 (1), pp. e85545.

Aries, A., Paradis, P., Lefebvre, C., Schwartz, R. J. & Nemer, M. (2004) 'Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity'. *Proc Natl Acad Sci U S A*, 101 (18), pp. 6975-6980.

Asgharian, B., Chen, Y. J., Patronas, N. J., Peghini, P. L., Reynolds, J. C., Vortmeyer, A., Zhuang, Z., Venzon, D. J., Gibril, F. & Jensen, R. T. (2004) 'Meningiomas may be a component tumor of multiple endocrine neoplasia type 1'. *Clin Cancer Res*, 10 (3), pp. 869-880.

Backer-Grondahl, T., Moen, B. H. & Torp, S. H. (2012) 'The histopathological spectrum of human meningiomas'. *Int J Clin Exp Pathol*, 5 (3), pp. 231-242.

Balar, A. V., Galsky, M. D., Rosenberg, J. E., Powles, T., Petrylak, D. P., Bellmunt, J., Loriot, Y., Necchi, A., Hoffman-Censits, J., Perez-Gracia, J. L., Dawson, N. A., van der Heijden, M. S., Dreicer, R., Srinivas, S., Retz, M. M., Joseph, R. W., Drakaki, A., Vaishampayan, U. N., Sridhar, S. S., Quinn, D. I., Duran, I., Shaffer, D. R., Eigl, B. J., Grivas, P. D., Yu, E. Y., Li, S., Kadel, E. E., 3rd, Boyd, Z., Bourgon, R., Hegde, P. S., Mariathasan, S., Thastrom, A., Abidoye, O. O., Fine, G. D., Bajorin, D. F. & Group, I. M. S. (2017) 'Atezolizumab as first-line treatment in cisplatin-ineligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial'. *Lancet*, 389 (10064), pp. 67-76.

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. & Draetta, G. (1993) 'Cyclin D1 is a nuclear protein required for cell cycle progression in G1'. *Genes Dev*, 7 (5), pp. 812-821.

Baskerville, S. & Bartel, D. P. (2005) 'Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes'. *RNA*, 11 (3), pp. 241-247.

Baumgarten, P., Gessler, F., Schittenhelm, J., Skardelly, M., Tews, D. S., Senft, C., Dunst, M., Imoehl, L., Plate, K. H., Wagner, M., Steinbach, J. P., Seifert, V., Mittelbronn, M. & Harter, P. N. (2016) 'Brain invasion in otherwise benign meningiomas does not predict tumor recurrence'. *Acta Neuropathol*, 132 (3), pp. 479-481.

Bayat Mokhtari, R., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B. & Yeger, H. (2017) 'Combination therapy in combating cancer'. *Oncotarget*, 8 (23), pp. 38022-38043.

Beauchamp, R., James, M., DeSouza, P., Wagh, V., Zhao, W., Jordan, J., Stemmer-Rachamimov, A., Plotkin, S., Gusella, J., Haggarty, S. & Ramesh, V. (2015) 'A high-throughput kinome screen reveals serum/glucocorticoid-regulated kinase 1 as a therapeutic target for NF2-deficient meningiomas.'. *Oncotarget*,

Beaver JA, G. J., Yi KH, Rajpurohit A, Thomas M, Gilbert SF, Rosen DM, Ho Park B, Lauring J. (2013) 'PIK3CA and AKT1 mutations have distinct effects on sensitivity to targeted pathway inhibitors in an isogenic luminal breast cancer model system.'. *Clin Cancer Res*, 19 (19), pp. 5413-5422.

Bebelman, M. P., Smit, M. J., Pegtel, D. M. & Baglio, S. R. (2018) 'Biogenesis and function of extracellular vesicles in cancer'. *Pharmacol Ther*, 188 pp. 1-11.

Bello, M. J., de Campos, J. M., Kusak, M. E., Vaquero, J., Sarasa, J. L., Pestana, A. & Rey, J. A. (1994) 'Allelic loss at 1p is associated with tumor progression of meningiomas'. *Genes Chromosomes Cancer*, 9 (4), pp. 296-298.

Benson, V., Pirie, K., Schüz, J., Reeves, G., Beral, V., Green, J. & Collaborators., M. W. S. (2013) 'Mobile phone use and risk of brain neoplasms and other cancers: prospective study.'. *Int J Epidemiol.*, 42 (3), pp. 792-802.

Bi, W. L., Greenwald, N. F., Abedalthagafi, M., Wala, J., Gibson, W. J., Agarwalla, P. K., Horowitz, P., Schumacher, S. E., Esaulova, E., Mei, Y., Chevalier, A., Ducar, M., Thorner, A. R., van Hummelen, P., Stemmer-Rachamimov, A., Artyomov, M., Al-Mefty, O., Dunn, G. P., Santagata, S., Dunn, I. F. & Beroukhim, R. (2017) 'Genomic landscape of high-grade meningiomas'. *NPJ Genom Med*, 2

Boetto, J., Apra, C., Bielle, F., Peyre, M. & Kalamarides, M. (2018) 'Selective vulnerability of the primitive meningeal layer to prenatal Smo activation for skull base meningothelial meningioma formation'. *Oncogene*, 37 (36), pp. 4955-4963.

Bostrom, J., Meyer-Puttlitz, B., Wolter, M., Blaschke, B., Weber, R. G., Lichter, P., Ichimura, K., Collins, V. P. & Reifenberger, G. (2001) 'Alterations of the tumor suppressor genes CDKN2A (p16(INK4a)), p14(ARF), CDKN2B

(p15(INK4b)), and CDKN2C (p18(INK4c)) in atypical and anaplastic meningiomas'. *Am J Pathol*, 159 (2), pp. 661-669.

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A. M., Schirle, M., Schlegl, J., Schwab, M., Stein, M. A., Bauer, A., Casari, G., Drewes, G., Gavin, A. C., Jackson, D. B., Joberty, G., Neubauer, G., Rick, J., Kuster, B. & Superti-Furga, G. (2004) 'A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway'. *Nat Cell Biol*, 6 (2), pp. 97-105.

Bradner, J. E., Hnisz, D. & Young, R. A. (2017) 'Transcriptional Addiction in Cancer'. *Cell*, 168 (4), pp. 629-643.

Braganza, M. Z., Kitahara, C. M., Berrington de Gonzalez, A., Inskip, P. D., Johnson, K. J. & Rajaraman, P. (2012) 'Ionizing radiation and the risk of brain and central nervous system tumors: a systematic review'. *Neuro Oncol*, 14 (11), pp. 1316-1324.

Brastianos, P. K., Galanis, E., Butowski, N., Chan, J. W., Dunn, I. F., Goldbrunner, R., Herold-Mende, C., Ippen, F. M., Mawrin, C., McDermott, M. W., Sloan, A., Snyder, J., Tabatabai, G., Tatagiba, M., Tonn, J. C., Wen, P. Y., Aldape, K., Nassiri, F., Zadeh, G., Jenkinson, M. D., Raleigh, D. R. & International Consortium on, M. (2019) 'Advances in multidisciplinary therapy for meningiomas'. *Neuro Oncol*, 21 (Supplement_1), pp. i18-i31.

Cagney, D. N., Sul, J., Huang, R. Y., Ligon, K. L., Wen, P. Y. & Alexander, B. M. (2018) 'The FDA NIH Biomarkers, EndpointS, and other Tools (BEST) resource in neuro-oncology'. *Neuro Oncol*, 20 (9), pp. 1162-1172.

Cai, D. X., Banerjee, R., Scheithauer, B. W., Lohse, C. M., Kleinschmidt-Demasters, B. K. & Perry, A. (2001) 'Chromosome 1p and 14q FISH analysis in clinicopathologic subsets of meningioma: diagnostic and prognostic implications'. *J Neuropathol Exp Neurol*, 60 (6), pp. 628-636.

Calboli, F. C., Cox, D. G., Buring, J. E., Gaziano, J. M., Ma, J., Stampfer, M., Willett, W. C., Tworoger, S. S., Hunter, D. J., Camargo, C. A., Jr. & Michaud, D. S. (2011) 'Prediagnostic plasma IgE levels and risk of adult glioma in four prospective cohort studies'. *J Natl Cancer Inst*, 103 (21), pp. 1588-1595.

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. & Croce, C. M. (2002) 'Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia'. *Proc Natl Acad Sci U S A*, 99 (24), pp. 15524-15529.

Camacho, L., Guerrero, P. & Marchetti, D. (2013) 'MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes'. *PLoS One*, 8 (9), pp. e73790.

Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., Hostetter, G., Boguslawski, S., Moses, T. Y., Savage, S., Uhlik, M., Lin, A.,

Du, J., Qian, Y. W., Zeckner, D. J., Tucker-Kellogg, G., Touchman, J., Patel, K., Mousses, S., Bittner, M., Schevitz, R., Lai, M. H., Blanchard, K. L. & Thomas, J. E. (2007) 'A transforming mutation in the pleckstrin homology domain of AKT1 in cancer'. *Nature*, 448 (7152), pp. 439-444.

Carter, D. R., Buckle, A. D., Tanaka, K., Perdomo, J. & Chong, B. H. (2014) 'Art27 interacts with GATA4, FOG2 and NKX2.5 and is a novel co-repressor of cardiac genes'. *PLoS One*, 9 (4), pp. e95253.

Carter, J. V., Pan, J., Rai, S. N. & Galandiuk, S. (2016) 'ROC-ing along: Evaluation and interpretation of receiver operating characteristic curves'. *Surgery*, 159 (6), pp. 1638-1645.

Carthew, R. W. & Sontheimer, E. J. (2009) 'Origins and Mechanisms of miRNAs and siRNAs'. *Cell*, 136 (4), pp. 642-655.

Caslini, C., Capo-chichi, C. D., Roland, I. H., Nicolas, E., Yeung, A. T. & Xu, X. X. (2006) 'Histone modifications silence the GATA transcription factor genes in ovarian cancer'. *Oncogene*, 25 (39), pp. 5446-5461.

Cha, D. J., Franklin, J. L., Dou, Y., Liu, Q., Higginbotham, J. N., Demory Beckler, M., Weaver, A. M., Vickers, K., Prasad, N., Levy, S., Zhang, B., Coffey, R. J. & Patton, J. G. (2015) 'KRAS-dependent sorting of miRNA to exosomes'. *Elife*, 4 pp. e07197.

Chamberlain, M. C. (2012) 'Hydroxyurea for recurrent surgery and radiation refractory high-grade meningioma'. *J Neurooncol*, 107 (2), pp. 315-321.

Chamoun, R., Krisht, K. M. & Couldwell, W. T. (2011) 'Incidental meningiomas'. *Neurosurg Focus*, 31 (6), pp. E19.

Chen, K. S., Lim, J. W. C., Richards, L. J. & Bunt, J. (2017a) 'The convergent roles of the nuclear factor I transcription factors in development and cancer'. *Cancer Lett*, 410 pp. 124-138.

Chen, S., Crabill, G. A., Pritchard, T. S., McMiller, T. L., Wei, P., Pardoll, D. M., Pan, F. & Topalian, S. L. (2019) 'Mechanisms regulating PD-L1 expression on tumor and immune cells'. *J Immunother Cancer*, 7 (1), pp. 305.

Chen, W. C., Magill, S. T., Englot, D. J., Baal, J. D., Wagle, S., Rick, J. W. & McDermott, M. W. (2017b) 'Factors Associated With Pre- and Postoperative Seizures in 1033 Patients Undergoing Supratentorial Meningioma Resection'. *Neurosurgery*, 81 (2), pp. 297-306.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J., Guo, X., Li, Q., Li, X., Wang, W., Zhang, Y., Wang, J., Jiang, X., Xiang, Y., Xu, C., Zheng, P., Zhang, J., Li, R., Zhang, H., Shang, X., Gong, T., Ning, G., Wang, J., Zen, K., Zhang, J. & Zhang, C. Y. (2008) 'Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases'. *Cell Res*, 18 (10), pp. 997-1006.

Cheng, G., Zhang, L., Lv, W., Dong, C., Wang, Y. & Zhang, J. (2015) 'Overexpression of cyclin D1 in meningioma is associated with malignancy grade and causes abnormalities in apoptosis, invasion and cell cycle progression'. *Med Oncol*, 32 (1), pp. 439.

Chia, N. Y., Deng, N., Das, K., Huang, D., Hu, L., Zhu, Y., Lim, K. H., Lee, M. H., Wu, J., Sam, X. X., Tan, G. S., Wan, W. K., Yu, W., Gan, A., Tan, A. L., Tay, S. T., Soo, K. C., Wong, W. K., Dominguez, L. T., Ng, H. H., Rozen, S., Goh, L. K., Teh, B. T. & Tan, P. (2015) 'Regulatory crosstalk between lineage-survival oncogenes KLF5, GATA4 and GATA6 cooperatively promotes gastric cancer development'. *Gut*, 64 (5), pp. 707-719.

Chiu, Y. L. & Rana, T. M. (2003) 'siRNA function in RNAi: a chemical modification analysis'. *RNA*, 9 (9), pp. 1034-1048.

Cho, J. A., Park, H., Lim, E. H. & Lee, K. W. (2012) 'Exosomes from breast cancer cells can convert adipose tissue-derived mesenchymal stem cells into myofibroblast-like cells'. *Int J Oncol*, 40 (1), pp. 130-138.

Chou, T. C. (2010) 'Drug combination studies and their synergy quantification using the Chou-Talalay method'. *Cancer Res*, 70 (2), pp. 440-446.

Chou, T. C. (2011) 'The mass-action law based algorithms for quantitative econo-green bio-research'. *Integr Biol (Camb)*, 3 (5), pp. 548-559.

Chow, H. Y., Dong, B., Duron, S. G., Campbell, D. A., Ong, C. C., Hoeflich, K. P., Chang, L. S., Welling, D. B., Yang, Z. J. & Chernoff, J. (2015) 'Group I Paks as therapeutic targets in NF2-deficient meningioma'. *Oncotarget*, 6 (4), pp. 1981-1994.

Choy, W., Kim, W., Nagasawa, D., Stramotas, S., Yew, A., Gopen, Q., Parsa, A. T. & Yang, I. (2011) 'The molecular genetics and tumor pathogenesis of meningiomas and the future directions of meningioma treatments'. *Neurosurg Focus*, 30 (5), pp. E6.

Clapier, C. R. & Cairns, B. R. (2009) 'The biology of chromatin remodeling complexes'. *Annu Rev Biochem*, 78 pp. 273-304.

Clark, V. E., Erson-Omay, E. Z., Serin, A., Yin, J., Cotney, J., Ozduman, K., Avsar, T., Li, J., Murray, P. B., Henegariu, O., Yilmaz, S., Gunel, J. M., Carrion-Grant, G., Yilmaz, B., Grady, C., Tanrikulu, B., Bakircioglu, M., Kaymakcalan, H., Caglayan, A. O., Sencar, L., Ceyhun, E., Atik, A. F., Bayri, Y., Bai, H., Kolb, L. E., Hebert, R. M., Omay, S. B., Mishra-Gorur, K., Choi, M., Overton, J. D., Holland, E. C., Mane, S., State, M. W., Bilguvar, K., Baehring, J. M., Gutin, P. H., Piepmeier, J. M., Vortmeyer, A., Brennan, C. W., Pamir, M. N., Kilic, T., Lifton, R. P., Noonan, J. P., Yasuno, K. & Gunel, M. (2013) 'Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO'. *Science*, 339 (6123), pp. 1077-1080.

Clark, V. E., Harmanci, A. S., Bai, H., Youngblood, M. W., Lee, T. I., Baranoski, J. F., Ercan-Sencicek, A. G., Abraham, B. J., Weintraub, A. S., Hnisz, D.,

Simon, M., Krischek, B., Erson-Omay, E. Z., Henegariu, O., Carrion-Grant, G., Mishra-Gorur, K., Duran, D., Goldmann, J. E., Schramm, J., Goldbrunner, R., Piepmeier, J. M., Vortmeyer, A. O., Gunel, J. M., Bilguvar, K., Yasuno, K., Young, R. A. & Gunel, M. (2016) 'Recurrent somatic mutations in POLR2A define a distinct subset of meningiomas'. *Nat Genet*, 48 (10), pp. 1253-1259.

Cocucci, E., Racchetti, G. & Meldolesi, J. (2009) 'Shedding microvesicles: artefacts no more'. *Trends Cell Biol*, 19 (2), pp. 43-51.

Collins, V. P. (2004) 'Brain tumours: classification and genes'. *J Neurol Neurosurg Psychiatry*, 75 Suppl 2 pp. ii2-11.

Colombo, M., Raposo, G. & Thery, C. (2014) 'Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles'. *Annu Rev Cell Dev Biol*, 30 pp. 255-289.

Combs, S. E., Ganswindt, U., Foote, R. L., Kondziolka, D. & Tonn, J. C. (2012) 'State-of-the-art treatment alternatives for base of skull meningiomas: complementing and controversial indications for neurosurgery, stereotactic and robotic based radiosurgery or modern fractionated radiation techniques'. *Radiat Oncol*, 7 pp. 226.

Commins, D. L., Atkinson, R. D. & Burnett, M. E. (2007) 'Review of meningioma histopathology'. *Neurosurg Focus*, 23 (4), pp. E3.

Cook, N. R. (2007) 'Use and misuse of the receiver operating characteristic curve in risk prediction'. *Circulation*, 115 (7), pp. 928-935.

Corcoran, D. L., Pandit, K. V., Gordon, B., Bhattacharjee, A., Kaminski, N. & Benos, P. V. (2009) 'Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data'. *PLoS One*, 4 (4), pp. e5279.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D. & Orkin, S. H. (2001) 'Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors'. *Genes Dev*, 15 (7), pp. 839-844.

Curto, M. & McClatchey, A. I. (2008) 'Nf2/Merlin: a coordinator of receptor signalling and intercellular contact'. *Br J Cancer*, 98 (2), pp. 256-262.

Custer, B., Longstreth, W. T., Jr., Phillips, L. E., Koepsell, T. D. & Van Belle, G. (2006) 'Hormonal exposures and the risk of intracranial meningioma in women: a population-based case-control study'. *BMC Cancer*, 6 pp. 152.

Daidone, M. G., Costa, A. & Silvestrini, R. (2001) 'Cell proliferation markers in human solid tumors: assessing their impact in clinical oncology'. *Methods Cell Biol*, 64 pp. 359-384.

de The, H., Chomienne, C., Lanotte, M., Degos, L. & Dejean, A. (1990) 'The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus'. *Nature*, 347 (6293), pp. 558-561.

Demetri, G. D., Chawla, S. P., von Mehren, M., Ritch, P., Baker, L. H., Blay, J. Y., Hande, K. R., Keohan, M. L., Samuels, B. L., Schuetze, S., Lebedinsky, C., Elsayed, Y. A., Izquierdo, M. A., Gomez, J., Park, Y. C. & Le Cesne, A. (2009) 'Efficacy and safety of trabectedin in patients with advanced or metastatic liposarcoma or leiomyosarcoma after failure of prior anthracyclines and ifosfamide: results of a randomized phase II study of two different schedules'. *J Clin Oncol*, 27 (25), pp. 4188-4196.

Derks, S., Postma, C., Moerkerk, P. T., van den Bosch, S. M., Carvalho, B., Hermsen, M. A., Giaretti, W., Herman, J. G., Weijenberg, M. P., de Bruine, A. P., Meijer, G. A. & van Engeland, M. (2006) 'Promoter methylation precedes chromosomal alterations in colorectal cancer development'. *Cell Oncol*, 28 (5-6), pp. 247-257.

Desterro, J. M., Rodriguez, M. S. & Hay, R. T. (2000) 'Regulation of transcription factors by protein degradation'. *Cell Mol Life Sci*, 57 (8-9), pp. 1207-1219.

Di Maio, S., Ramanathan, D., Garcia-Lopez, R., Rocha, M. H., Guerrero, F. P., Ferreira, M., Jr. & Sekhar, L. N. (2012) 'Evolution and future of skull base surgery: the paradigm of skull base meningiomas'. *World Neurosurg*, 78 (3-4), pp. 260-275.

Dinkova-Kostova, A. T., Kostov, R. V. & Kazantsev, A. G. (2018) 'The role of Nrf2 signaling in counteracting neurodegenerative diseases'. *FEBS J*, 285 (19), pp. 3576-3590.

Du, X., Lin, L. I., Zhang, L. & Jiang, J. (2015a) 'microRNA-195 inhibits the proliferation, migration and invasion of cervical cancer cells via the inhibition of CCND2 and MYB expression'. *Oncol Lett*, 10 (4), pp. 2639-2643.

Du, Z., Abedalthagafi, M., Aizer, A. A., McHenry, A. R., Sun, H. H., Bray, M. A., Viramontes, O., Machaidze, R., Brastianos, P. K., Reardon, D. A., Dunn, I. F., Freeman, G. J., Ligon, K. L., Carpenter, A. E., Alexander, B. M., Agar, N. Y., Rodig, S. J., Bradshaw, E. M. & Santagata, S. (2015b) 'Increased expression of the immune modulatory molecule PD-L1 (CD274) in anaplastic meningioma'. *Oncotarget*, 6 (7), pp. 4704-4716.

Du, Z., Tong, X. & Ye, X. (2013) 'Cyclin D1 promotes cell cycle progression through enhancing NDR1/2 kinase activity independent of cyclin-dependent kinase 4'. *J Biol Chem*, 288 (37), pp. 26678-26687.

Duan, Y. & Chen, Q. (2016) 'TGF-beta1 regulating miR-205/miR-195 expression affects the TGF-beta signal pathway by respectively targeting SMAD2/SMAD7'. *Oncol Rep*, 36 (4), pp. 1837-1844.

Duffy, M. J., Synnott, N. C. & Crown, J. (2017) 'Mutant p53 as a target for cancer treatment'. *Eur J Cancer*, 83 pp. 258-265.

Dunn, J., Ferluga, S., Sharma, V., Futschik, M., Hilton, D. A., Adams, C. L., Lasonder, E. & Hanemann, C. O. (2019) 'Proteomic analysis discovers the differential expression of novel proteins and phosphoproteins in meningioma

including NEK9, HK2 and SET and deregulation of RNA metabolism'. *EBioMedicine*, 40 pp. 77-91.

El-Gewely, M. R., Andreassen, M., Walquist, M., Ursvik, A., Knutsen, E., Nystad, M., Coucheron, D. H., Myrmel, K. S., Hennig, R. & Johansen, S. D. (2016) 'Differentially Expressed MicroRNAs in Meningiomas Grades I and II Suggest Shared Biomarkers with Malignant Tumors'. *Cancers (Basel)*, 8 (3),

El-Hachem, N. & Nemer, G. (2011) 'Identification of new GATA4-small molecule inhibitors by structure-based virtual screening'. *Bioorg Med Chem*, 19 (5), pp. 1734-1742.

Endoh-Yamagami, S., Evangelista, M., Wilson, D., Wen, X., Theunissen, J. W., Phamluong, K., Davis, M., Scales, S. J., Solloway, M. J., de Sauvage, F. J. & Peterson, A. S. (2009) 'The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development'. *Curr Biol*, 19 (15), pp. 1320-1326.

Englot, D. J., Magill, S. T., Han, S. J., Chang, E. F., Berger, M. S. & McDermott, M. W. (2016) 'Seizures in supratentorial meningioma: a systematic review and meta-analysis'. *J Neurosurg*, 124 (6), pp. 1552-1561.

Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G. J., Zanesi, N., Crawford, M., Ozer, G. H., Wernicke, D., Alder, H., Caligiuri, M. A., Nana-Sinkam, P., Perrotti, D. & Croce,

C. M. (2012) 'MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response'. *Proc Natl Acad Sci U S A*, 109 (31), pp. E2110-2116.

Fang L, L. D., Meizlish ML, Anderson RC, Bruce JN, Devine L, Huttner AJ, Kleinstein SH, Lee JY, Stern JN, Yaari G, Lovato L, Cronk KM, O'Connor KC. (2013) 'The immune cell infiltrate populating meningiomas is composed of mature, antigen-experienced T and B cells.'. *Neuro Oncol.*, 15 (11), pp. 1479-1490.

Fani, M., Nicolas, G. P. & Wild, D. (2017) 'Somatostatin Receptor Antagonists for Imaging and Therapy'. *J Nucl Med*, 58 (Suppl 2), pp. 61S-66S.

Farra, R., Dapas, B., Baiz, D., Tonon, F., Chiaretti, S., Del Sal, G., Rustighi, A., Elvassore, N., Pozzato, G., Grassi, M. & Grassi, G. (2015) 'Impairment of the Pin1/E2F1 axis in the anti-proliferative effect of bortezomib in hepatocellular carcinoma cells'. *Biochimie*, 112 pp. 85-95.

Finn, R. S., Crown, J. P., Lang, I., Boer, K., Bondarenko, I. M., Kulyk, S. O., Ettl, J., Patel, R., Pinter, T., Schmidt, M., Shparyk, Y., Thummala, A. R., Voytko, N. L., Fowst, C., Huang, X., Kim, S. T., Randolph, S. & Slamon, D. J. (2015) 'The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study'. *Lancet Oncol*, 16 (1), pp. 25-35.

Finn, R. S., Dering, J., Conklin, D., Kalous, O., Cohen, D. J., Desai, A. J., Ginther, C., Atefi, M., Chen, I., Fowst, C., Los, G. & Slamon, D. J. (2009) 'PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro'. *Breast Cancer Res*, 11 (5), pp. R77.

Finn, R. S., Martin, M., Rugo, H. S., Jones, S., Im, S. A., Gelmon, K., Harbeck, N., Lipatov, O. N., Walshe, J. M., Moulder, S., Gauthier, E., Lu, D. R., Randolph, S., Dieras, V. & Slamon, D. J. (2016) 'Palbociclib and Letrozole in Advanced Breast Cancer'. *N Engl J Med*, 375 (20), pp. 1925-1936.

Fishilevich, S., Nudel, R., Rappaport, N., Hadar, R., Plaschkes, I., Iny Stein, T., Rosen, N., Kohn, A., Twik, M., Safran, M., Lancet, D. & Cohen, D. (2017) 'GeneHancer: genome-wide integration of enhancers and target genes in GeneCards'. *Database (Oxford)*, 2017

Fontaine, B., Rouleau, G. A., Seizinger, B. R., Menon, A. G., Jewell, A. F., Martuza, R. L. & Gusella, J. F. (1991) 'Molecular genetics of neurofibromatosis 2 and related tumors (acoustic neuroma and meningioma)'. *Ann N Y Acad Sci*, 615 pp. 338-343.

Foucquier, J. & Guedj, M. (2015) 'Analysis of drug combinations: current methodological landscape'. *Pharmacol Res Perspect*, 3 (3), pp. e00149.

Fouladi, M., Perentesis, J. P., Phillips, C. L., Leary, S., Reid, J. M., McGovern, R. M., Ingle, A. M., Ahern, C. H., Ames, M. M., Houghton, P., Doyle, L. A.,

Weigel, B. & Blaney, S. M. (2014) 'A phase I trial of MK-2206 in children with refractory malignancies: a Children's Oncology Group study'. *Pediatr Blood Cancer*, 61 (7), pp. 1246-1251.

Fredly, H., Gjertsen, B. T. & Bruserud, O. (2013) 'Histone deacetylase inhibition in the treatment of acute myeloid leukemia: the effects of valproic acid on leukemic cells, and the clinical and experimental evidence for combining valproic acid with other antileukemic agents'. *Clin Epigenetics*, 5 (1), pp. 12.

Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. (2009) 'Most mammalian mRNAs are conserved targets of microRNAs'. *Genome Res*, 19 (1), pp. 92-105.

Fu, M., Wang, C., Li, Z., Sakamaki, T. & Pestell, R. G. (2004) 'Minireview: Cyclin D1: normal and abnormal functions'. *Endocrinology*, 145 (12), pp. 5439-5447.

Fu, M., Wang, C., Rao, M., Wu, X., Bouras, T., Zhang, X., Li, Z., Jiao, X., Yang, J., Li, A., Perkins, N. D., Thimmapaya, B., Kung, A. L., Munoz, A., Giordano, A., Lisanti, M. P. & Pestell, R. G. (2005) 'Cyclin D1 represses p300 transactivation through a cyclin-dependent kinase-independent mechanism'. *J Biol Chem*, 280 (33), pp. 29728-29742.

Furuta, M., Kozaki, K., Tanimoto, K., Tanaka, S., Arii, S., Shimamura, T., Niida, A., Miyano, S. & Inazawa, J. (2013) 'The tumor-suppressive miR-497-195

cluster targets multiple cell-cycle regulators in hepatocellular carcinoma'. *PLoS One*, 8 (3), pp. e60155.

Gallo, A., Tandon, M., Alevizos, I. & Illei, G. G. (2012) 'The majority of microRNAs detectable in serum and saliva is concentrated in exosomes'. *PLoS One*, 7 (3), pp. e30679.

Gao, P., Zhou, G. Y., Liu, Y., Li, J. S., Zhen, J. H. & Yuan, Y. P. (2004) 'Alteration of cyclin D1 in gastric carcinoma and its clinicopathologic significance'. *World J Gastroenterol*, 10 (20), pp. 2936-2939.

Garcia-Manero, G., Yang, H., Bueso-Ramos, C., Ferrajoli, A., Cortes, J., Wierda, W. G., Faderl, S., Koller, C., Morris, G., Rosner, G., Loboda, A., Fantin, V. R., Randolph, S. S., Hardwick, J. S., Reilly, J. F., Chen, C., Ricker, J. L., Secrist, J. P., Richon, V. M., Frankel, S. R. & Kantarjian, H. M. (2008) 'Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes'. *Blood*, 111 (3), pp. 1060-1066.

Gay, E., Lages, E., Ramus, C., Guttin, A., El Atifi, M., Dupre, I., Bouamrani, A., Salon, C., Ratel, D., Wion, D., Berger, F. & Issartel, J. P. (2011) 'The heterogeneity of meningioma revealed by multiparameter analysis: infiltrative and non-infiltrative clinical phenotypes'. *Int J Oncol*, 38 (5), pp. 1287-1297.

Gerber, D. E., Grossman, S. A., Zeltzman, M., Parisi, M. A. & Kleinberg, L. (2007) 'The impact of thrombocytopenia from temozolomide and radiation in newly diagnosed adults with high-grade gliomas'. *Neuro Oncol*, 9 (1), pp. 47-52.

Giles, A. J., Hao, S., Padget, M. R., Song, H., Zhang, W., Lynes, J., Sanchez, V. E., Liu, Y., Jung, J., Cao, X., Fujii, R., Jensen, R. L., Gillespie, D., Schlom, J., Gilbert, M. R., Nduom, E. K., Yang, C., Lee, J. H., Soon-Shiong, P., Hodge, J. W. & Park, D. M. (2019) 'Efficient ADCC- killing of meningioma by avelumab and a high-affinity natural killer cell line, haNK'. *JCI Insight*,

Gogineni, V. R., Gupta, R., Nalla, A. K., Velpula, K. K. & Rao, J. S. (2012) 'uPAR and cathepsin B shRNA impedes TGF-beta1-driven proliferation and invasion of meningioma cells in a XIAP-dependent pathway'. *Cell Death Dis*, 3 pp. e439.

Goldbrunner, R., Minniti, G., Preusser, M., Jenkinson, M. D., Sallabanda, K., Houdart, E., von Deimling, A., Stavrinou, P., Lefranc, F., Lund-Johansen, M., Moyal, E. C., Brandsma, D., Henriksson, R., Soffietti, R. & Weller, M. (2016) 'EANO guidelines for the diagnosis and treatment of meningiomas'. *Lancet Oncol*, 17 (9), pp. e383-391.

Goldie, B. J., Dun, M. D., Lin, M., Smith, N. D., Verrills, N. M., Dayas, C. V. & Cairns, M. J. (2014) 'Activity-associated miRNA are packaged in Map1b-enriched exosomes released from depolarized neurons'. *Nucleic Acids Res*, 42 (14), pp. 9195-9208.

Gomez-Pastor, R., Burchfiel, E. T. & Thiele, D. J. (2018) 'Regulation of heat shock transcription factors and their roles in physiology and disease'. *Nat Rev Mol Cell Biol*, 19 (1), pp. 4-19.

Goutagny, S., Bah, A. B., Henin, D., Parfait, B., Grayeli, A. B., Sterkers, O. & Kalamarides, M. (2012) 'Long-term follow-up of 287 meningiomas in neurofibromatosis type 2 patients: clinical, radiological, and molecular features'. *Neuro Oncol*, 14 (8), pp. 1090-1096.

Goutagny, S., Nault, J. C., Mallet, M., Henin, D., Rossi, J. Z. & Kalamarides, M. (2014) 'High incidence of activating TERT promoter mutations in meningiomas undergoing malignant progression'. *Brain Pathol*, 24 (2), pp. 184-189.

Goutagny, S., Yang, H. W., Zucman-Rossi, J., Chan, J., Dreyfuss, J. M., Park, P. J., Black, P. M., Giovannini, M., Carroll, R. S. & Kalamarides, M. (2010) 'Genomic profiling reveals alternative genetic pathways of meningioma malignant progression dependent on the underlying NF2 status'. *Clin Cancer Res*, 16 (16), pp. 4155-4164.

Griffiths, S. G., Cormier, M. T., Clayton, A. & Doucette, A. A. (2017) 'Differential Proteome Analysis of Extracellular Vesicles from Breast Cancer Cell Lines by Chaperone Affinity Enrichment'. *Proteomes*, 5 (4),

Guarducci, C., Bonechi, M., Benelli, M., Biagioni, C., Boccalini, G., Romagnoli, D., Verardo, R., Schiff, R., Osborne, C. K., De Angelis, C., Di Leo, A., Malorni, L. & Migliaccio, I. (2018) 'Cyclin E1 and Rb modulation as common events at

time of resistance to palbociclib in hormone receptor-positive breast cancer'. *NPJ Breast Cancer*, 4 pp. 38.

Guduric-Fuchs, J., O'Connor, A., Camp, B., O'Neill, C. L., Medina, R. J. & Simpson, D. A. (2012) 'Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types'. *BMC Genomics*, 13 pp. 357.

Guo, M., Akiyama, Y., House, M. G., Hooker, C. M., Heath, E., Gabrielson, E., Yang, S. C., Han, Y., Baylin, S. B., Herman, J. G. & Brock, M. V. (2004) 'Hypermethylation of the GATA genes in lung cancer'. *Clin Cancer Res*, 10 (23), pp. 7917-7924.

Guo, M., House, M. G., Akiyama, Y., Qi, Y., Capagna, D., Harmon, J., Baylin, S. B., Brock, M. V. & Herman, J. G. (2006) 'Hypermethylation of the GATA gene family in esophageal cancer'. *Int J Cancer*, 119 (9), pp. 2078-2083.

Gutmann, D. H., Donahoe, J., Perry, A., Lemke, N., Gorse, K., Kittiniyom, K., Rempel, S. A., Gutierrez, J. A. & Newsham, I. F. (2000) 'Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas'. *Hum Mol Genet*, 9 (10), pp. 1495-1500.

Gyorgy, B., Szabo, T. G., Pasztoi, M., Pal, Z., Misjak, P., Aradi, B., Laszlo, V., Pallinger, E., Pap, E., Kittel, A., Nagy, G., Falus, A. & Buzas, E. I. (2011) 'Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles'. *Cell Mol Life Sci*, 68 (16), pp. 2667-2688.

Hajian-Tilaki, K. (2013) 'Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation'. *Caspian J Intern Med*, 4 (2), pp. 627-635.

Haley, B. & Zamore, P. D. (2004) 'Kinetic analysis of the RNAi enzyme complex'. *Nat Struct Mol Biol*, 11 (7), pp. 599-606.

Hallinan, J. T., Hegde, A. N. & Lim, W. E. (2013) 'Dilemmas and diagnostic difficulties in meningioma'. *Clin Radiol*, 68 (8), pp. 837-844.

Han, M. S., Kim, Y. J., Moon, K. S., Lee, K. H., Yang, J. I., Kang, W. D., Lim, S. H., Jang, W. Y., Jung, T. Y., Kim, I. Y. & Jung, S. (2016a) 'Lessons from surgical outcome for intracranial meningioma involving major venous sinus'. *Medicine (Baltimore)*, 95 (35), pp. e4705.

Han, S. J., Reis, G., Kohanbash, G., Shrivastav, S., Magill, S. T., Molinaro, A. M., McDermott, M. W., Theodosopoulos, P. V., Aghi, M. K., Berger, M. S., Butowski, N. A., Barani, I., Phillips, J. J., Perry, A. & Okada, H. (2016b) 'Expression and prognostic impact of immune modulatory molecule PD-L1 in meningioma'. *J Neurooncol*, 130 (3), pp. 543-552.

Hanley, J. A. & McNeil, B. J. (1982) 'The meaning and use of the area under a receiver operating characteristic (ROC) curve'. *Radiology*, 143 (1), pp. 29-36.

He, Z., Ruan, X., Liu, X., Zheng, J., Liu, Y., Liu, L., Ma, J., Shao, L., Wang, D., Shen, S., Yang, C. & Xue, Y. (2019) 'FUS/circ_002136/miR-138-5p/SOX13 feedback loop regulates angiogenesis in Glioma'. *J Exp Clin Cancer Res*, 38 (1), pp. 65.

Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J. & Sixma, J. J. (1999) 'Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules'. *Blood*, 94 (11), pp. 3791-3799.

Hellebrekers, D. M., Lentjes, M. H., van den Bosch, S. M., Melotte, V., Wouters, K. A., Daenen, K. L., Smits, K. M., Akiyama, Y., Yuasa, Y., Sanduleanu, S., Khalid-de Bakker, C. A., Jonkers, D., Weijenberg, M. P., Louwagie, J., van Criekinge, W., Carvalho, B., Meijer, G. A., Baylin, S. B., Herman, J. G., de Bruine, A. P. & van Engeland, M. (2009) 'GATA4 and GATA5 are potential tumor suppressors and biomarkers in colorectal cancer'. *Clin Cancer Res*, 15 (12), pp. 3990-3997.

Holleczek, B., Zampella, D., Urbschat, S., Sahm, F., von Deimling, A., Oertel, J. & Ketter, R. (2019) 'Incidence, mortality and outcome of meningiomas: A population-based study from Germany'. *Cancer Epidemiol*, 62 pp. 101562.

Hou, L., Zhu, L., Li, H., Jiang, F., Cao, L., Hu, C. Y. & Wang, C. (2019) 'MiR-501-3p Forms a Feedback Loop with FOS, MDFI, and MyoD to Regulate C2C12 Myogenesis'. *Cells*, 8 (6),

Huang, X., Yuan, T., Tschannen, M., Sun, Z., Jacob, H., Du, M., Liang, M., Dittmar, R. L., Liu, Y., Liang, M., Kohli, M., Thibodeau, S. N., Boardman, L. & Wang, L. (2013) 'Characterization of human plasma-derived exosomal RNAs by deep sequencing'. *BMC Genomics*, 14 pp. 319.

Hui, W., Yuntao, L., Lun, L., WenSheng, L., ChaoFeng, L., HaiYong, H. & Yueyang, B. (2013) 'MicroRNA-195 inhibits the proliferation of human glioma cells by directly targeting cyclin D1 and cyclin E1'. *PLoS One*, 8 (1), pp. e54932.

Hutvagner, G. & Zamore, P. D. (2002) 'A microRNA in a multiple-turnover RNAi enzyme complex'. *Science*, 297 (5589), pp. 2056-2060.

Hwang, J. Y. & Zukin, R. S. (2018) 'REST, a master transcriptional regulator in neurodegenerative disease'. *Curr Opin Neurobiol*, 48 pp. 193-200.

Hydbring, P., Malumbres, M. & Sicinski, P. (2016) 'Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases'. *Nat Rev Mol Cell Biol*, 17 (5), pp. 280-292.

Hyman, D. M., Smyth, L. M., Donoghue, M. T. A., Westin, S. N., Bedard, P. L., Dean, E. J., Bando, H., El-Khoueiry, A. B., Perez-Fidalgo, J. A., Mita, A., Schellens, J. H. M., Chang, M. T., Reichel, J. B., Bouvier, N., Selcuklu, S. D., Soumerai, T. E., Torrisi, J., Erinjeri, J. P., Ambrose, H., Barrett, J. C., Dougherty, B., Foxley, A., Lindemann, J. P. O., McEwen, R., Pass, M., Schiavon, G., Berger, M. F., Chandarlapaty, S., Solit, D. B., Banerji, U.,

Baselga, J. & Taylor, B. S. (2017) 'AKT Inhibition in Solid Tumors With AKT1 Mutations'. *J Clin Oncol*, 35 (20), pp. 2251-2259.

Imjeti, N. S., Menck, K., Egea-Jimenez, A. L., Lecointre, C., Lembo, F., Bouguenina, H., Badache, A., Ghossoub, R., David, G., Roche, S. & Zimmermann, P. (2017) 'Syntenin mediates SRC function in exosomal cell-to-cell communication'. *Proc Natl Acad Sci U S A*, 114 (47), pp. 12495-12500.

Iplikcioglu, A. C., Hatiboglu, M. A., Ozek, E. & Ozcan, D. (2014) 'Is progesteron receptor status really a prognostic factor for intracranial meningiomas?'. *Clin Neurol Neurosurg*, 124 pp. 119-122.

Jaiswal, J. K., Andrews, N. W. & Simon, S. M. (2002) 'Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells'. *J Cell Biol*, 159 (4), pp. 625-635.

James, M. F., Han, S., Polizzano, C., Plotkin, S. R., Manning, B. D., Stemmer-Rachamimov, A. O., Gusella, J. F. & Ramesh, V. (2009) 'NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth'. *Mol Cell Biol*, 29 (15), pp. 4250-4261.

James, M. F., Stivison, E., Beauchamp, R., Han, S., Li, H., Wallace, M. R., Gusella, J. F., Stemmer-Rachamimov, A. O. & Ramesh, V. (2012) 'Regulation of mTOR complex 2 signaling in neurofibromatosis 2-deficient target cell types'. *Mol Cancer Res*, 10 (5), pp. 649-659.

Jenkins, R. B., Xiao, Y., Sicotte, H., Decker, P. A., Kollmeyer, T. M., Hansen, H. M., Kosel, M. L., Zheng, S., Walsh, K. M., Rice, T., Bracci, P., McCoy, L. S., Smirnov, I., Patoka, J. S., Hsuang, G., Wiemels, J. L., Tihan, T., Pico, A. R., Prados, M. D., Chang, S. M., Berger, M. S., Caron, A. A., Fink, S. R., Halder, C., Rynearson, A. L., Fridley, B. L., Buckner, J. C., O'Neill, B. P., Giannini, C., Lachance, D. H., Wiencke, J. K., Eckel-Passow, J. E. & Wrensch, M. R. (2012) 'A low-frequency variant at 8q24.21 is strongly associated with risk of oligodendroglial tumors and astrocytomas with IDH1 or IDH2 mutation'. *Nat Genet*, 44 (10), pp. 1122-1125.

Jenkinson, M. D., Javadpour, M., Haylock, B. J., Young, B., Gillard, H., Vinten, J., Bulbeck, H., Das, K., Farrell, M., Looby, S., Hickey, H., Preusser, M., Mallucci, C. L., Hughes, D., Gamble, C. & Weber, D. C. (2015) 'The ROAM/EORTC-1308 trial: Radiation versus Observation following surgical resection of Atypical Meningioma: study protocol for a randomised controlled trial'. *Trials*, 16 pp. 519.

Kadoch, C. & Crabtree, G. R. (2015) 'Mammalian SWI/SNF chromatin remodeling complexes and cancer: Mechanistic insights gained from human genomics'. *Sci Adv*, 1 (5), pp. e1500447.

Kahlert, C. & Kalluri, R. (2013) 'Exosomes in tumor microenvironment influence cancer progression and metastasis'. *J Mol Med (Berl)*, 91 (4), pp. 431-437.

Kaley, T. J., Wen, P., Schiff, D., Ligon, K., Haidar, S., Karimi, S., Lassman, A. B., Nolan, C. P., DeAngelis, L. M., Gavrilovic, I., Norden, A., Drappatz, J., Lee, E. Q., Purow, B., Plotkin, S. R., Batchelor, T., Abrey, L. E. & Omuro, A. (2015) 'Phase II trial of sunitinib for recurrent and progressive atypical and anaplastic meningioma'. *Neuro Oncol*, 17 (1), pp. 116-121.

Kalluri, R. (2016) 'The biology and function of exosomes in cancer'. *J Clin Invest*, 126 (4), pp. 1208-1215.

Kanada, M., Bachmann, M. H., Hardy, J. W., Frimannson, D. O., Bronsart, L., Wang, A., Sylvester, M. D., Schmidt, T. L., Kaspar, R. L., Butte, M. J., Matin, A. C. & Contag, C. H. (2015) 'Differential fates of biomolecules delivered to target cells via extracellular vesicles'. *Proc Natl Acad Sci U S A*, 112 (12), pp. E1433-1442.

Kaye, S. B., Colombo, N., Monk, B. J., Tjulandin, S., Kong, B., Roy, M., Chan, S., Filipczyk-Cisarz, E., Hagberg, H., Vergote, I., Lebedinsky, C., Parekh, T., Santabarbara, P., Park, Y. C., Nieto, A. & Poveda, A. (2011) 'Trabectedin plus pegylated liposomal doxorubicin in relapsed ovarian cancer delays third-line chemotherapy and prolongs the platinum-free interval'. *Ann Oncol*, 22 (1), pp. 49-58.

Ketter, R., Kim, Y. J., Storck, S., Rahnenfuhrer, J., Romeike, B. F., Steudel, W. I., Zang, K. D. & Henn, W. (2007) 'Hyperdiploidy defines a distinct cytogenetic entity of meningiomas'. *J Neurooncol*, 83 (2), pp. 213-221.

Kijima, C., Miyashita, T., Suzuki, M., Oka, H. & Fujii, K. (2012) 'Two cases of nevoid basal cell carcinoma syndrome associated with meningioma caused by a PTCH1 or SUFU germline mutation'. *Fam Cancer*, 11 (4), pp. 565-570.

Kim, V. N. (2005) 'MicroRNA biogenesis: coordinated cropping and dicing'. *Nat Rev Mol Cell Biol*, 6 (5), pp. 376-385.

King, H. W., Michael, M. Z. & Gleadle, J. M. (2012) 'Hypoxic enhancement of exosome release by breast cancer cells'. *BMC Cancer*, 12 pp. 421.

Kliese, N., Gobrecht, P., Pachow, D., Andrae, N., Wilisch-Neumann, A., Kirches, E., Riek-Burchardt, M., Angenstein, F., Reifenberger, G., Riemenschneider, M., Meese, E., Panayotova-Dimitrova, D., Gutmann, D. & Mawrin, C. (2013) 'miRNA-145 is downregulated in atypical and anaplastic meningiomas and negatively regulates motility and proliferation of meningioma cells.'. *Oncogene*, 32 (39), pp. 4712-4720.

Kobayashi, S., Volden, P., Timm, D., Mao, K., Xu, X. & Liang, Q. (2010) 'Transcription factor GATA4 inhibits doxorubicin-induced autophagy and cardiomyocyte death'. *J Biol Chem*, 285 (1), pp. 793-804.

Korhonen, K., Salminen, T., Raitanen, J., Auvinen, A., Isola, J. & Haapasalo, H. (2006) 'Female predominance in meningiomas can not be explained by differences in progesterone, estrogen, or androgen receptor expression'. *J Neurooncol*, 80 (1), pp. 1-7.

Kosaka, N., Iguchi, H. & Ochiya, T. (2010) 'Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis'. *Cancer Sci*, 101 (10), pp. 2087-2092.

Kostareli, E., Holzinger, D., Bogatyrova, O., Hielscher, T., Wichmann, G., Keck, M., Lahrmann, B., Grabe, N., Flechtenmacher, C., Schmidt, C. R., Seiwert, T., Dyckhoff, G., Dietz, A., Hofler, D., Pawlita, M., Benner, A., Bosch, F. X., Plinkert, P., Plass, C., Weichenhan, D. & Hess, J. (2013) 'HPV-related methylation signature predicts survival in oropharyngeal squamous cell carcinomas'. *J Clin Invest*, 123 (6), pp. 2488-2501.

Kyronlahti, A., Ramo, M., Tamminen, M., Unkila-Kallio, L., Butzow, R., Leminen, A., Nemer, M., Rahman, N., Huhtaniemi, I., Heikinheimo, M. & Anttonen, M. (2008) 'GATA-4 regulates Bcl-2 expression in ovarian granulosa cell tumors'. *Endocrinology*, 149 (11), pp. 5635-5642.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. (2001) 'Identification of novel genes coding for small expressed RNAs'. *Science*, 294 (5543), pp. 853-858.

Laitinen, M. P., Anttonen, M., Ketola, I., Wilson, D. B., Ritvos, O., Butzow, R. & Heikinheimo, M. (2000) 'Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors'. *J Clin Endocrinol Metab*, 85 (9), pp. 3476-3483.

Lambert, M., Jambon, S., Depauw, S. & David-Cordonnier, M. H. (2018) 'Targeting Transcription Factors for Cancer Treatment'. *Molecules*, 23 (6),

Lamszus, K., Kluwe, L., Matschke, J., Meissner, H., Laas, R. & Westphal, M. (1999) 'Allelic losses at 1p, 9q, 10q, 14q, and 22q in the progression of aggressive meningiomas and undifferentiated meningeal sarcomas'. *Cancer Genet Cytogenet*, 110 (2), pp. 103-110.

Lasser, C., Alikhani, V. S., Ekstrom, K., Eldh, M., Paredes, P. T., Bossios, A., Sjostrand, M., Gabrielsson, S., Lotvall, J. & Valadi, H. (2011) 'Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages'. *J Transl Med*, 9 pp. 9.

Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. (2001) 'An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans'. *Science*, 294 (5543), pp. 858-862.

Lawrie, C. H., Gal, S., Dunlop, H. M., Pushkaran, B., Liggins, A. P., Pulford, K., Banham, A. H., Pezzella, F., Boultwood, J., Wainscoat, J. S., Hatton, C. S. & Harris, A. L. (2008) 'Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma'. *Br J Haematol*, 141 (5), pp. 672-675.

Lawson, C., Vicencio, J. M., Yellon, D. M. & Davidson, S. M. (2016) 'Microvesicles and exosomes: new players in metabolic and cardiovascular disease'. *J Endocrinol*, 228 (2), pp. R57-71.

Lee, R. C. & Ambros, V. (2001) 'An extensive class of small RNAs in Caenorhabditis elegans'. *Science*, 294 (5543), pp. 862-864.

Lee, T. H., Chennakrishnaiah, S., Audemard, E., Montermini, L., Meehan, B. & Rak, J. (2014) 'Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells'. *Biochem Biophys Res Commun*, 451 (2), pp. 295-301.

Lehar, J., Zimmermann, G. R., Krueger, A. S., Molnar, R. A., Ledell, J. T., Heilbut, A. M., Short, G. F., 3rd, Giusti, L. C., Nolan, G. P., Magid, O. A., Lee, M. S., Borisy, A. A., Stockwell, B. R. & Keith, C. T. (2007) 'Chemical combination effects predict connectivity in biological systems'. *Mol Syst Biol*, 3 pp. 80.

Lentjes, M. H., Niessen, H. E., Akiyama, Y., de Bruine, A. P., Melotte, V. & van Engeland, M. (2016) 'The emerging role of GATA transcription factors in development and disease'. *Expert Rev Mol Med*, 18 pp. e3.

Lewis, B. P., Burge, C. B. & Bartel, D. P. (2005) 'Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets'. *Cell*, 120 (1), pp. 15-20.

Li, D., Zhao, Y., Liu, C., Chen, X., Qi, Y., Jiang, Y., Zou, C., Zhang, X., Liu, S., Wang, X., Zhao, D., Sun, Q., Zeng, Z., Dress, A., Lin, M. C., Kung, H. F., Rui, H., Liu, L. Z., Mao, F., Jiang, B. H. & Lai, L. (2011) 'Analysis of MiR-195 and

MiR-497 expression, regulation and role in breast cancer'. *Clin Cancer Res*, 17 (7), pp. 1722-1730.

Li, L., Li, C., Wang, S., Wang, Z., Jiang, J., Wang, W., Li, X., Chen, J., Liu, K., Li, C. & Zhu, G. (2016) 'Exosomes Derived from Hypoxic Oral Squamous Cell Carcinoma Cells Deliver miR-21 to Normoxic Cells to Elicit a Prometastatic Phenotype'. *Cancer Res*, 76 (7), pp. 1770-1780.

Lilly, A. J., Lacaud, G. & Kouskoff, V. (2017) 'SOXF transcription factors in cardiovascular development'. *Semin Cell Dev Biol*, 63 pp. 50-57.

Lin, L., Aggarwal, S., Glover, T. W., Orringer, M. B., Hanash, S. & Beer, D. G. (2000) 'A minimal critical region of the 8p22-23 amplicon in esophageal adenocarcinomas defined using sequence tagged site-amplification mapping and quantitative polymerase chain reaction includes the GATA-4 gene'. *Cancer Res*, 60 (5), pp. 1341-1347.

Lindholm, P. M., Soini, Y., Myllarniemi, M., Knuutila, S., Heikinheimo, M., Kinnula, V. L. & Salmenkivi, K. (2009) 'Expression of GATA-6 transcription factor in pleural malignant mesothelioma and metastatic pulmonary adenocarcinoma'. *J Clin Pathol*, 62 (4), pp. 339-344.

Link, W. & Fernandez-Marcos, P. J. (2017) 'FOXO transcription factors at the interface of metabolism and cancer'. *Int J Cancer*, 141 (12), pp. 2379-2391.

Linos, E., Raine, T., Alonso, A. & Michaud, D. (2007) 'Atopy and risk of brain tumors: a meta-analysis'. *J Natl Cancer Inst*, 99 (20), pp. 1544-1550.

Linsler, S., Kraemer, D., Driess, C., Oertel, J., Kammers, K., Rahnenfuhrer, J., Ketter, R. & Urbschat, S. (2014) 'Molecular biological determinations of meningioma progression and recurrence'. *PLoS One*, 9 (4), pp. e94987.

Liu, J., Shen, J. X., Wen, X. F., Guo, Y. X. & Zhang, G. J. (2016) 'Targeting Notch degradation system provides promise for breast cancer therapeutics'. *Crit Rev Oncol Hematol*, 104 pp. 21-29.

Livak, K. J. & Schmittgen, T. D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method'. *Methods*, 25 (4), pp. 402-408.

Lou, E., Sumrall, A. L., Turner, S., Peters, K. B., Desjardins, A., Vredenburgh, J. J., McLendon, R. E., Herndon, J. E., 2nd, McSherry, F., Norfleet, J., Friedman, H. S. & Reardon, D. A. (2012) 'Bevacizumab therapy for adults with recurrent/progressive meningioma: a retrospective series'. *J Neurooncol*, 109 (1), pp. 63-70.

Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., Ohgaki, H., Wiestler, O. D., Kleihues, P. & Ellison, D. W. (2016) 'The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary'. *Acta Neuropathol*, 131 (6), pp. 803-820.

Louis, D. N. & von Deimling, A. (1995) 'Hereditary tumor syndromes of the nervous system: overview and rare syndromes'. *Brain Pathol*, 5 (2), pp. 145-151.

Ludwig, N., Kim, Y., Mueller, S., Backes, C., Werner, T., Galata, V., Sartorius, E., Bohle, R., Keller, A. & Meese, E. (2015) 'Posttranscriptional deregulation of signaling pathways in meningioma subtypes by differential expression of miRNAs.'. *Neuro Oncol.*, 17 (9), pp. 1250-1260.

Lundberg, A. S. & Weinberg, R. A. (1999) 'Control of the cell cycle and apoptosis'. *Eur J Cancer*, 35 (14), pp. 1886-1894.

Luo, Q., Zhang, Z., Dai, Z., Basnet, S., Li, S., Xu, B. & Ge, H. (2016) 'Tumor-suppressive microRNA-195-5p regulates cell growth and inhibits cell cycle by targeting cyclin dependent kinase 8 in colon cancer'. *Am J Transl Res*, 8 (5), pp. 2088-2096.

Lusis, E. A., Scheithauer, B. W., Yachnis, A. T., Fischer, B. R., Chicoine, M. R., Paulus, W. & Perry, A. (2012) 'Meningiomas in pregnancy: a clinicopathologic study of 17 cases'. *Neurosurgery*, 71 (5), pp. 951-961.

Ma, R., Jiang, T. & Kang, X. (2012) 'Circulating microRNAs in cancer: origin, function and application'. *J Exp Clin Cancer Res*, 31 pp. 38.

Mark, J., Levan, G. & Mitelman, F. (1972) 'Identification by fluorescence of the G chromosome lost in human meningomas'. *Hereditas*, 71 (1), pp. 163-168.

Mark, J., Mitelman, F. & Levan, G. (1972) 'On the specificity of the G abnormality in human meningomas studied by the fluorescence technique'. *Acta Pathol Microbiol Scand A*, 80 (6), pp. 812-820.

Marosi, C., Hassler, M., Roessler, K., Reni, M., Sant, M., Mazza, E. & Vecht, C. (2008) 'Meningioma'. *Crit Rev Oncol Hematol*, 67 (2), pp. 153-171.

Martin, P., Bartlett, N. L., Blum, K. A., Park, S., Maddocks, K., Ruan, J., Ridling, L., Dittus, C., Chen, Z., Huang, X., Inghirami, G., DiLiberto, M., Chen-Kiang, S. & Leonard, J. P. (2019) 'A phase 1 trial of ibrutinib plus palbociclib in previously treated mantle cell lymphoma'. *Blood*, 133 (11), pp. 1201-1204.

Massard, C., Gordon, M. S., Sharma, S., Rafii, S., Wainberg, Z. A., Luke, J., Curiel, T. J., Colon-Otero, G., Hamid, O., Sanborn, R. E., O'Donnell, P. H., Drakaki, A., Tan, W., Kurland, J. F., Rebelatto, M. C., Jin, X., Blake-Haskins, J. A., Gupta, A. & Segal, N. H. (2016) 'Safety and Efficacy of Durvalumab (MEDI4736), an Anti-Programmed Cell Death Ligand-1 Immune Checkpoint Inhibitor, in Patients With Advanced Urothelial Bladder Cancer'. *J Clin Oncol*, 34 (26), pp. 3119-3125.

Matsuyama, H. & Suzuki, H. I. (2019) 'Systems and Synthetic microRNA Biology: From Biogenesis to Disease Pathogenesis'. *Int J Mol Sci*, 21 (1),

Mawrin, C., Chung, C. & Preusser, M. (2015) 'Biology and clinical management challenges in meningioma'. *Am Soc Clin Oncol Educ Book*, pp. e106-115.

Melin, B. S., Barnholtz-Sloan, J. S., Wrensch, M. R., Johansen, C., Il'yasova, D., Kinnersley, B., Ostrom, Q. T., Labreche, K., Chen, Y., Armstrong, G., Liu, Y., Eckel-Passow, J. E., Decker, P. A., Labussiere, M., Idbaih, A., Hoang-Xuan, K., Di Stefano, A. L., Mokhtari, K., Delattre, J. Y., Broderick, P., Galan, P., Gousias, K., Schramm, J., Schoemaker, M. J., Fleming, S. J., Herms, S., Heilmann, S., Nothen, M. M., Wichmann, H. E., Schreiber, S., Swerdlow, A., Lathrop, M., Simon, M., Sanson, M., Andersson, U., Rajaraman, P., Chanock, S., Linet, M., Wang, Z., Yeager, M., GliomaScan, C., Wiencke, J. K., Hansen, H., McCoy, L., Rice, T., Kosel, M. L., Sicotte, H., Amos, C. I., Bernstein, J. L., Davis, F., Lachance, D., Lau, C., Merrell, R. T., Shildkraut, J., Ali-Osman, F., Sadetzki, S., Scheurer, M., Shete, S., Lai, R. K., Claus, E. B., Olson, S. H., Jenkins, R. B., Houlston, R. S. & Bondy, M. L. (2017) 'Genome-wide association study of glioma subtypes identifies specific differences in genetic susceptibility to glioblastoma and non-glioblastoma tumors'. *Nat Genet*, 49 (5), pp. 789-794.

Meyyappan, M., Wong, H., Hull, C. & Riabowol, K. T. (1998) 'Increased expression of cyclin D2 during multiple states of growth arrest in primary and established cells'. *Mol Cell Biol*, 18 (6), pp. 3163-3172.

Milenkovic, S., Marinkovic, T., Jovanovic, M. B., Djuricic, S., Berisavac, Il & Berisavac, I. (2008) 'Cyclin D1 immunoreactivity in meningiomas'. *Cell Mol Neurobiol*, 28 (6), pp. 907-913.

Min, A., Kim, J. E., Kim, Y. J., Lim, J. M., Kim, S., Kim, J. W., Lee, K. H., Kim, T. Y., Oh, D. Y., Bang, Y. J. & Im, S. A. (2018) 'Cyclin E overexpression confers

resistance to the CDK4/6 specific inhibitor palbociclib in gastric cancer cells'. *Cancer Lett*, 430 pp. 123-132.

Misra, B. K. (2012) 'The paradigm of skull base meningiomas: what is optimal?'. *World Neurosurg*, 78 (3-4), pp. 220-221.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. & Tewari, M. (2008) 'Circulating microRNAs as stable blood-based markers for cancer detection'. *Proc Natl Acad Sci U S A*, 105 (30), pp. 10513-10518.

Motekallemi, A., Jeltema, H. R., Metzemaekers, J. D., van Dam, G. M., Crane, L. M. & Groen, R. J. (2015) 'The current status of 5-ALA fluorescence-guided resection of intracranial meningiomas-a critical review'. *Neurosurg Rev*, 38 (4), pp. 619-628.

Mulcahy, L. A., Pink, R. C. & Carter, D. R. (2014) 'Routes and mechanisms of extracellular vesicle uptake'. *J Extracell Vesicles*, 3

Munich, S., Sobo-Vujanovic, A., Buchser, W. J., Beer-Stolz, D. & Vujanovic, N. L. (2012) 'Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands'. *Oncoimmunology*, 1 (7), pp. 1074-1083.

Nayeri, A., Chotai, S., Prablek, M. A., Brinson, P. R., Douleh, D. G., Weaver, K. D., Thompson, R. C. & Chambless, L. (2016) 'Type 2 diabetes is an independent negative prognostic factor in patients undergoing surgical resection of a WHO grade I meningioma'. *Clin Neurol Neurosurg*, 149 pp. 6-10.

Norden, A. D., Raizer, J. J., Abrey, L. E., Lamborn, K. R., Lassman, A. B., Chang, S. M., Yung, W. K., Gilbert, M. R., Fine, H. A., Mehta, M., Deangelis, L. M., Cloughesy, T. F., Robins, H. I., Aldape, K., Dancey, J., Prados, M. D., Lieberman, F. & Wen, P. Y. (2010) 'Phase II trials of erlotinib or gefitinib in patients with recurrent meningioma'. *J Neurooncol*, 96 (2), pp. 211-217.

Nunes, F., Shen, Y., Niida, Y., Beauchamp, R., Stemmer-Rachamimov, A. O., Ramesh, V., Gusella, J. & MacCollin, M. (2005) 'Inactivation patterns of NF2 and DAL-1/4.1B (EPB41L3) in sporadic meningioma'. *Cancer Genet Cytogenet*, 162 (2), pp. 135-139.

Nunes FP, M. V., Jennings D, Caruso PA, di Tomaso E, Muzikansky A, Barker FG 2nd, Stemmer-Rachamimov A, Plotkin SR. (2013) 'Bevacizumab treatment for meningiomas in NF2: a retrospective analysis of 15 patients.'. *PLoS One*, 8 (3),

O'Carroll, D. & Schaefer, A. (2013) 'General principals of miRNA biogenesis and regulation in the brain'. *Neuropsychopharmacology*, 38 (1), pp. 39-54.

Ogata-Kawata, H., Izumiya, M., Kurioka, D., Honma, Y., Yamada, Y., Furuta, K., Gunji, T., Ohta, H., Okamoto, H., Sonoda, H., Watanabe, M., Nakagama, H.,

Yokota, J., Kohno, T. & Tsuchiya, N. (2014) 'Circulating exosomal microRNAs as biomarkers of colon cancer'. *PLoS One*, 9 (4), pp. e92921.

Ohgaki, H. & Kleihues, P. (2005) 'Epidemiology and etiology of gliomas'. *Acta Neuropathol*, 109 (1), pp. 93-108.

Olar, A., Wani, K. M., Wilson, C. D., Zadeh, G., DeMonte, F., Jones, D. T., Pfister, S. M., Sulman, E. P. & Aldape, K. D. (2017) 'Global epigenetic profiling identifies methylation subgroups associated with recurrence-free survival in meningioma'. *Acta Neuropathol*, 133 (3), pp. 431-444.

Ostrom, Q. T., Gittleman, H., Fulop, J., Liu, M., Blanda, R., Kromer, C., Wolinsky, Y., Kruchko, C. & Barnholtz-Sloan, J. S. (2015) 'CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008-2012'. *Neuro Oncol*, 17 Suppl 4 pp. iv1-iv62.

Ostrom, Q. T., Gittleman, H., Truitt, G., Boscia, A., Kruchko, C. & Barnholtz-Sloan, J. S. (2018) 'CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2011-2015'. *Neuro Oncol*, 20 (suppl_4), pp. iv1-iv86.

Pachow, D., Andrae, N., Kliese, N., Angenstein, F., Stork, O., Wilisch-Neumann, A., Kirches, E. & Mawrin, C. (2013) 'mTORC1 inhibitors suppress meningioma growth in mouse models'. *Clin Cancer Res*, 19 (5), pp. 1180-1189.

Pajtler, K. W., Witt, H., Sill, M., Jones, D. T., Hovestadt, V., Kratochwil, F., Wani, K., Tatevossian, R., Punchihewa, C., Johann, P., Reimand, J., Warnatz, H. J., Ryzhova, M., Mack, S., Ramaswamy, V., Capper, D., Schweizer, L., Sieber, L., Wittmann, A., Huang, Z., van Sluis, P., Volckmann, R., Koster, J., Versteeg, R., Fults, D., Toledano, H., Avigad, S., Hoffman, L. M., Donson, A. M., Foreman, N., Hewer, E., Zitterbart, K., Gilbert, M., Armstrong, T. S., Gupta, N., Allen, J. C., Karajannis, M. A., Zagzag, D., Hasselblatt, M., Kulozik, A. E., Witt, O., Collins, V. P., von Hoff, K., Rutkowski, S., Pietsch, T., Bader, G., Yaspo, M. L., von Deimling, A., Lichter, P., Taylor, M. D., Gilbertson, R., Ellison, D. W., Aldape, K., Korshunov, A., Kool, M. & Pfister, S. M. (2015) 'Molecular Classification of Ependymal Tumors across All CNS Compartments, Histopathological Grades, and Age Groups'. *Cancer Cell*, 27 (5), pp. 728-743.

Pan, B. T. & Johnstone, R. (1984) 'Selective externalization of the transferrin receptor by sheep reticulocytes in vitro. Response to ligands and inhibitors of endocytosis'. *J Biol Chem*, 259 (15), pp. 9776-9782.

Pan, B. T. & Johnstone, R. M. (1983) 'Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor'. *Cell*, 33 (3), pp. 967-978.

Pan, B. T., Teng, K., Wu, C., Adam, M. & Johnstone, R. M. (1985) 'Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes'. *J Cell Biol*, 101 (3), pp. 942-948.

Perry, A., Giannini, C., Raghavan, R., Scheithauer, B. W., Banerjee, R., Margraf, L., Bowers, D. C., Lytle, R. A., Newsham, I. F. & Gutmann, D. H. (2001) 'Aggressive phenotypic and genotypic features in pediatric and NF2-associated meningiomas: a clinicopathologic study of 53 cases'. *J Neuropathol Exp Neurol*, 60 (10), pp. 994-1003.

Perry, A., Gutmann, D. H. & Reifenberger, G. (2004) 'Molecular pathogenesis of meningiomas'. *J Neurooncol*, 70 (2), pp. 183-202.

Peters, I., Dubrowinskaja, N., Kogosov, M., Abbas, M., Hennenlotter, J., von Klot, C., Merseburger, A. S., Stenzl, A., Scherer, R., Kuczyk, M. A. & Serth, J. (2014a) 'Decreased GATA5 mRNA expression associates with CpG island methylation and shortened recurrence-free survival in clear cell renal cell carcinoma'. *BMC Cancer*, 14 pp. 101.

Peters, I., Gebauer, K., Dubrowinskaja, N., Atschekzei, F., Kramer, M. W., Hennenlotter, J., Tezval, H., Abbas, M., Scherer, R., Merseburger, A. S., Stenzl, A., Kuczyk, M. A. & Serth, J. (2014b) 'GATA5 CpG island hypermethylation is an independent predictor for poor clinical outcome in renal cell carcinoma'. *Oncol Rep*, 31 (4), pp. 1523-1530.

Petrilli, A. M. & Fernandez-Valle, C. (2016) 'Role of Merlin/NF2 inactivation in tumor biology'. *Oncogene*, 35 (5), pp. 537-548.

Pike, B. L., Greiner, T. C., Wang, X., Weisenburger, D. D., Hsu, Y. H., Renaud, G., Wolfsberg, T. G., Kim, M., Weisenberger, D. J., Siegmund, K. D., Ye, W.,

Groshen, S., Mehrian-Shai, R., Delabie, J., Chan, W. C., Laird, P. W. & Hacia, J. G. (2008) 'DNA methylation profiles in diffuse large B-cell lymphoma and their relationship to gene expression status'. *Leukemia*, 22 (5), pp. 1035-1043.

Polvani, S., Tarocchi, M., Tempesti, S., Bencini, L. & Galli, A. (2016) 'Peroxisome proliferator activated receptors at the crossroad of obesity, diabetes, and pancreatic cancer'. *World J Gastroenterol*, 22 (8), pp. 2441-2459.

Poon, R. Y. (2016) 'Cell Cycle Control: A System of Interlinking Oscillators'. *Methods Mol Biol*, 1342 pp. 3-19.

Porrello, E. R., Johnson, B. A., Aurora, A. B., Simpson, E., Nam, Y. J., Matkovich, S. J., Dorn, G. W., 2nd, van Rooij, E. & Olson, E. N. (2011) 'MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes'. *Circ Res*, 109 (6), pp. 670-679.

Porta, C., Paglino, C. & Mosca, A. (2014) 'Targeting PI3K/Akt/mTOR Signaling in Cancer'. *Front Oncol*, 4 pp. 64.

Powles, T., Eder, J. P., Fine, G. D., Braiteh, F. S., Loriot, Y., Cruz, C., Bellmunt, J., Burris, H. A., Petrylak, D. P., Teng, S. L., Shen, X., Boyd, Z., Hegde, P. S., Chen, D. S. & Vogelzang, N. J. (2014) 'MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer'. *Nature*, 515 (7528), pp. 558-562.

Preusser, M., Brastianos, P. K. & Mawrin, C. (2018) 'Advances in meningioma genetics: novel therapeutic opportunities'. *Nat Rev Neurol*, 14 (2), pp. 106-115.

Preusser, M., Spiegl-Kreinecker, S., Lotsch, D., Wohrer, A., Schmook, M., Dieckmann, K., Saringer, W., Marosi, C. & Berger, W. (2012) 'Trabectedin has promising antineoplastic activity in high-grade meningioma'. *Cancer*, 118 (20), pp. 5038-5049.

Puttmann, S., Senner, V., Braune, S., Hillmann, B., Exeler, R., Rickert, C. H. & Paulus, W. (2005) 'Establishment of a benign meningioma cell line by hTERT-mediated immortalization'. *Lab Invest*, 85 (9), pp. 1163-1171.

Qiu, S., Lin, S., Hu, D., Feng, Y., Tan, Y. & Peng, Y. (2013) 'Interactions of miR-323/miR-326/miR-329 and miR-130a/miR-155/miR-210 as prognostic indicators for clinical outcome of glioblastoma patients'. *J Transl Med*, 11 pp. 10.

Quan, X., Li, X., Yin, Z., Ren, Y. & Zhou, B. (2019) 'p53/miR-30a-5p/ SOX4 feedback loop mediates cellular proliferation, apoptosis, and migration of non-small-cell lung cancer'. *J Cell Physiol*, 234 (12), pp. 22884-22895.

Rabinowits, G., Gercel-Taylor, C., Day, J. M., Taylor, D. D. & Kloecker, G. H. (2009) 'Exosomal microRNA: a diagnostic marker for lung cancer'. *Clin Lung Cancer*, 10 (1), pp. 42-46.

Rachinger, W., Stoecklein, V. M., Terpolilli, N. A., Haug, A. R., Ertl, L., Poschl, J., Schuller, U., Schichor, C., Thon, N. & Tonn, J. C. (2015) 'Increased 68Ga-

DOTATATE uptake in PET imaging discriminates meningioma and tumor-free tissue'. *J Nucl Med*, 56 (3), pp. 347-353.

Rahmatullah, M., Schroering, A., Rothblum, K., Stahl, R. C., Urban, B. & Carey, D. J. (1998) 'Synergistic regulation of Schwann cell proliferation by heregulin and forskolin'. *Mol Cell Biol*, 18 (11), pp. 6245-6252.

Raizer, J. J., Grimm, S. A., Rademaker, A., Chandler, J. P., Muro, K., Helenowski, I., Rice, L., McCarthy, K., Johnston, S. K., Mrugala, M. M. & Chamberlain, M. (2014) 'A phase II trial of PTK787/ZK 222584 in recurrent or progressive radiation and surgery refractory meningiomas'. *J Neurooncol*, 117 (1), pp. 93-101.

Ramteke, A., Ting, H., Agarwal, C., Mateen, S., Somasagara, R., Hussain, A., Graner, M., Frederick, B., Agarwal, R. & Deep, G. (2015) 'Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules'. *Mol Carcinog*, 54 (7), pp. 554-565.

Rana, S., Kumar, R., Sultana, S. & Sharma, R. K. (2010) 'Radiation-induced biomarkers for the detection and assessment of absorbed radiation doses'. *J Pharm Bioallied Sci*, 2 (3), pp. 189-196.

Raposo, G. & Stoorvogel, W. (2013) 'Extracellular vesicles: exosomes, microvesicles, and friends'. *J Cell Biol*, 200 (4), pp. 373-383.

Ravikumar, G. & Ananthamurthy, A. (2014) 'Cyclin D1 expression in ductal carcinoma of the breast and its correlation with other prognostic parameters'. *J Cancer Res Ther*, 10 (3), pp. 671-675.

Riemenschneider, M. J., Perry, A. & Reifenberger, G. (2006) 'Histological classification and molecular genetics of meningiomas'. *Lancet Neurol*, 5 (12), pp. 1045-1054.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. (2004) 'Identification of mammalian microRNA host genes and transcription units'. *Genome Res*, 14 (10A), pp. 1902-1910.

Rogers, C. L., Perry, A., Pugh, S., Vogelbaum, M. A., Brachman, D., McMillan, W., Jenrette, J., Barani, I., Shrieve, D., Sloan, A., Bovi, J., Kwok, Y., Burri, S. H., Chao, S. T., Spalding, A. C., Anscher, M. S., Bloom, B. & Mehta, M. (2016) 'Pathology concordance levels for meningioma classification and grading in NRG Oncology RTOG Trial 0539'. *Neuro Oncol*, 18 (4), pp. 565-574.

Rogers, L., Barani, I., Chamberlain, M., Kaley, T. J., McDermott, M., Raizer, J., Schiff, D., Weber, D. C., Wen, P. Y. & Vogelbaum, M. A. (2015) 'Meningiomas: knowledge base, treatment outcomes, and uncertainties. A RANO review'. *J Neurosurg*, 122 (1), pp. 4-23.

Rojas, A., Kong, S. W., Agarwal, P., Gilliss, B., Pu, W. T. & Black, B. L. (2008) 'GATA4 is a direct transcriptional activator of cyclin D2 and Cdk4 and is

required for cardiomyocyte proliferation in anterior heart field-derived myocardium'. *Mol Cell Biol*, 28 (17), pp. 5420-5431.

Rossi, L., Biagioni, C., McCartney, A., Migliaccio, I., Curigliano, G., Sanna, G., Moretti, E., Minisini, A. M., Cinieri, S., Tondini, C., Arpino, G., Bernardo, A., Martignetti, A., Risi, E., Pestrin, M., Boni, L., Benelli, M., Biganzoli, L., Di Leo, A. & Malorni, L. (2019) 'Clinical outcomes after palbociclib with or without endocrine therapy in postmenopausal women with hormone receptor positive and HER2-negative metastatic breast cancer enrolled in the TREnd trial'. *Breast Cancer Res*, 21 (1), pp. 71.

Rouleau, G. A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B. & et al. (1993) 'Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2'. *Nature*, 363 (6429), pp. 515-521.

Sahm, F., Schrimpf, D., Olar, A., Koelsche, C., Reuss, D., Bissel, J., Kratz, A., Capper, D., Schefzyk, S., Hielscher, T., Wang, Q., Sulman, E. P., Adeberg, S., Koch, A., Okuducu, A. F., Brehmer, S., Schittenhelm, J., Becker, A., Brokinkel, B., Schmidt, M., Ull, T., Gousias, K., Kessler, A. F., Lamszus, K., Debus, J., Mawrin, C., Kim, Y. J., Simon, M., Ketter, R., Paulus, W., Aldape, K. D., Herold-Mende, C. & von Deimling, A. (2016) 'TERT Promoter Mutations and Risk of Recurrence in Meningioma'. *J Natl Cancer Inst*, 108 (5),

Sahm, F., Schrimpf, D., Stichel, D., Jones, D. T. W., Hielscher, T., Schefzyk, S., Okonechnikov, K., Koelsche, C., Reuss, D. E., Capper, D., Sturm, D.,

Wirsching, H. G., Berghoff, A. S., Baumgarten, P., Kratz, A., Huang, K., Wefers, A. K., Hovestadt, V., Sill, M., Ellis, H. P., Kurian, K. M., Okuducu, A. F., Jungk, C., Drueschler, K., Schick, M., Bewerunge-Hudler, M., Mawrin, C., Seiz-Rosenhagen, M., Ketter, R., Simon, M., Westphal, M., Lamszus, K., Becker, A., Koch, A., Schittenhelm, J., Rushing, E. J., Collins, V. P., Brehmer, S., Chavez, L., Platten, M., Hanggi, D., Unterberg, A., Paulus, W., Wick, W., Pfister, S. M., Mittelbronn, M., Preusser, M., Herold-Mende, C., Weller, M. & von Deimling, A. (2017) 'DNA methylation-based classification and grading system for meningioma: a multicentre, retrospective analysis'. *Lancet Oncol*, 18 (5), pp. 682-694.

Sato-Kuwabara, Y., Melo, S. A., Soares, F. A. & Calin, G. A. (2015) 'The fusion of two worlds: non-coding RNAs and extracellular vesicles--diagnostic and therapeutic implications (Review)'. *Int J Oncol*, 46 (1), pp. 17-27.

Saydam, O., Shen, Y., Wurdinger, T., Senol, O., Boke, E., James, M. F., Tannous, B. A., Stemmer-Rachamimov, A. O., Yi, M., Stephens, R. M., Fraefel, C., Gusella, J. F., Krichevsky, A. M. & Breakefield, X. O. (2009) 'Downregulated microRNA-200a in meningiomas promotes tumor growth by reducing E-cadherin and activating the Wnt/beta-catenin signaling pathway'. *Mol Cell Biol*, 29 (21), pp. 5923-5940.

Schichor, C., Terpolilli, N., Thorsteinsdottir, J. & Tonn, J. C. (2017) 'Intraoperative Computed Tomography in Cranial Neurosurgery'. *Neurosurg Clin N Am*, 28 (4), pp. 595-602.

Schito, L. & Semenza, G. L. (2016) 'Hypoxia-Inducible Factors: Master Regulators of Cancer Progression'. *Trends Cancer*, 2 (12), pp. 758-770.

Schmitz, U., Mueller, W., Weber, M., Sevenet, N., Delattre, O. & von Deimling, A. (2001) 'INI1 mutations in meningiomas at a potential hotspot in exon 9'. *Br J Cancer*, 84 (2), pp. 199-201.

Schneider, B., Pulhorn, H., Rohrig, B. & Rainov, N. G. (2005) 'Predisposing conditions and risk factors for development of symptomatic meningioma in adults'. *Cancer Detect Prev*, 29 (5), pp. 440-447.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis'. *Nat Methods*, 9 (7), pp. 671-675.

Schwartzbaum, J. A., Fisher, J. L., Aldape, K. D. & Wrensch, M. (2006) 'Epidemiology and molecular pathology of glioma'. *Nat Clin Pract Neurol*, 2 (9), pp. 494-503; quiz 491 p following 516.

Senol, O., Schaaij-Visser, T. B., Erkan, E. P., Dorfer, C., Lewandrowski, G., Pham, T. V., Piersma, S. R., Peerdeman, S. M., Strobel, T., Tannous, B., Saydam, N., Slavc, I., Knosp, E., Jimenez, C. R. & Saydam, O. (2015) 'miR-200a-mediated suppression of non-muscle heavy chain IIb inhibits meningioma cell migration and tumor growth in vivo'. *Oncogene*, 34 (14), pp. 1790-1798.

Shah, N. R., Tancioni, I., Ward, K. K., Lawson, C., Chen, X. L., Jean, C., Sulzmaier, F. J., Uryu, S., Miller, N. L., Connolly, D. C. & Schlaepfer, D. D.

(2014) 'Analyses of merlin/NF2 connection to FAK inhibitor responsiveness in serous ovarian cancer'. *Gynecol Oncol*, 134 (1), pp. 104-111.

Shao, H., Chung, J., Balaj, L., Charest, A., Bigner, D. D., Carter, B. S., Hochberg, F. H., Breakefield, X. O., Weissleder, R. & Lee, H. (2012) 'Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy'. *Nat Med*, 18 (12), pp. 1835-1840.

Shapiro, I. M., Kolev, V. N., Vidal, C. M., Kadariya, Y., Ring, J. E., Wright, Q., Weaver, D. T., Menges, C., Padval, M., McClatchey, A. I., Xu, Q., Testa, J. R. & Pachter, J. A. (2014) 'Merlin deficiency predicts FAK inhibitor sensitivity: a synthetic lethal relationship'. *Sci Transl Med*, 6 (237), pp. 237ra268.

Sharabi, A. B., Lim, M., DeWeese, T. L. & Drake, C. G. (2015) 'Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy'. *Lancet Oncol*, 16 (13), pp. e498-509.

Shih, K. C., Chowdhary, S., Rosenblatt, P., Weir, A. B., 3rd, Shepard, G. C., Williams, J. T., Shastry, M., Burris, H. A., 3rd & Hainsworth, J. D. (2016) 'A phase II trial of bevacizumab and everolimus as treatment for patients with refractory, progressive intracranial meningioma'. *J Neurooncol*, 129 (2), pp. 281-288.

Silapunt, S., Chen, L. & Migden, M. R. (2016) 'Hedgehog pathway inhibition in advanced basal cell carcinoma: latest evidence and clinical usefulness'. *Ther Adv Med Oncol*, 8 (5), pp. 375-382.

Silva, J., Garcia, V., Zaballos, A., Provencio, M., Lombardia, L., Almonacid, L., Garcia, J. M., Dominguez, G., Pena, C., Diaz, R., Herrera, M., Varela, A. & Bonilla, F. (2011) 'Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival'. *Eur Respir J*, 37 (3), pp. 617-623.

Simon, M., von Deimling, A., Larson, J. J., Wellenreuther, R., Kaskel, P., Waha, A., Warnick, R. E., Tew, J. M., Jr. & Menon, A. G. (1995) 'Allelic losses on chromosomes 14, 10, and 1 in atypical and malignant meningiomas: a genetic model of meningioma progression'. *Cancer Res*, 55 (20), pp. 4696-4701.

Simons, M. & Raposo, G. (2009) 'Exosomes--vesicular carriers for intercellular communication'. *Curr Opin Cell Biol*, 21 (4), pp. 575-581.

Simpson, D. (1957) 'The recurrence of intracranial meningiomas after surgical treatment'. *J Neurol Neurosurg Psychiatry*, 20 (1), pp. 22-39.

Sindou, M. P. & Alvernia, J. E. (2006) 'Results of attempted radical tumor removal and venous repair in 100 consecutive meningiomas involving the major dural sinuses'. *J Neurosurg*, 105 (4), pp. 514-525.

Skapek, S. X., Rhee, J., Kim, P. S., Novitch, B. G. & Lassar, A. B. (1996) 'Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation'. *Mol Cell Biol*, 16 (12), pp. 7043-7053.

Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr., Carter, B. S., Krichevsky, A. M. & Breakefield, X. O. (2008) 'Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers'. *Nat Cell Biol*, 10 (12), pp. 1470-1476.

Slomka, A., Urban, S. K., Lukacs-Kornek, V., Zekanowska, E. & Kornek, M. (2018) 'Large Extracellular Vesicles: Have We Found the Holy Grail of Inflammation?'. *Front Immunol*, 9 pp. 2723.

Smalheiser, N. R., Lugli, G., Torvik, V. I., Mise, N., Ikeda, R. & Abe, K. (2008) 'Natural antisense transcripts are co-expressed with sense mRNAs in synaptoneurosomes of adult mouse forebrain'. *Neurosci Res*, 62 (4), pp. 236-239.

Smith, M. J. (2015) 'Germline and somatic mutations in meningiomas'. *Cancer Genet*, 208 (4), pp. 107-114.

Smith MJ, H. J., Bowers NL, Halliday D, Paterson J, Gillespie J, Huson SM, Freeman SR, Lloyd S, Rutherford SA, King AT, Wallace AJ, Ramsden RT, Evans DG. (2011) 'Cranial meningiomas in 411 neurofibromatosis type 2 (NF2) patients with proven gene mutations: clear positional effect of mutations, but absence of female severity effect on age at onset.'. *J Med Genet.*, 48 (4), pp. 261-265.

Smith, M. J., O'Sullivan, J., Bhaskar, S. S., Hadfield, K. D., Poke, G., Caird, J., Sharif, S., Eccles, D., Fitzpatrick, D., Rawluk, D., du Plessis, D., Newman, W. G. & Evans, D. G. (2013) 'Loss-of-function mutations in SMARCE1 cause an inherited disorder of multiple spinal meningiomas'. *Nat Genet*, 45 (3), pp. 295-298.

Smith, M. J., Wallace, A. J., Bennett, C., Hasselblatt, M., Elert-Dobkowska, E., Evans, L. T., Hickey, W. F., van Hoff, J., Bauer, D., Lee, A., Hevner, R. F., Beetz, C., du Plessis, D., Kilday, J. P., Newman, W. G. & Evans, D. G. (2014) 'Germline SMARCE1 mutations predispose to both spinal and cranial clear cell meningiomas'. *J Pathol*, 234 (4), pp. 436-440.

Smith, M. J., Wallace, A. J., Bowers, N. L., Rustad, C. F., Woods, C. G., Leschziner, G. D., Ferner, R. E. & Evans, D. G. (2012) 'Frequency of SMARCB1 mutations in familial and sporadic schwannomatosis'. *Neurogenetics*, 13 (2), pp. 141-145.

Song, Y., Tian, T., Fu, X., Wang, W., Li, S., Shi, T., Suo, A., Ruan, Z., Guo, H. & Yao, Y. (2015) 'GATA6 is overexpressed in breast cancer and promotes breast cancer cell epithelial-mesenchymal transition by upregulating slug expression'. *Exp Mol Pathol*, 99 (3), pp. 617-627.

Srinivasan, M. & Lahiri, D. K. (2015) 'Significance of NF-kappaB as a pivotal therapeutic target in the neurodegenerative pathologies of Alzheimer's disease and multiple sclerosis'. *Expert Opin Ther Targets*, 19 (4), pp. 471-487.

Sur, I. & Taipale, J. (2016) 'The role of enhancers in cancer'. *Nat Rev Cancer*, 16 (8), pp. 483-493.

Takahashi, K. & Yamanaka, S. (2006) 'Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors'. *Cell*, 126 (4), pp. 663-676.

Takasugi, M., Okada, R., Takahashi, A., Virya Chen, D., Watanabe, S. & Hara, E. (2017) 'Small extracellular vesicles secreted from senescent cells promote cancer cell proliferation through EphA2'. *Nat Commun*, 8 pp. 15729.

Tang, G. (2005) 'siRNA and miRNA: an insight into RISCs'. *Trends Biochem Sci*, 30 (2), pp. 106-114.

Taylor, D. D. & Gercel-Taylor, C. (2008) 'MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer'. *Gynecol Oncol*, 110 (1), pp. 13-21.

Taylor, D. D. & Shah, S. (2015) 'Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes'. *Methods*, 87 pp. 3-10.

Terpolilli, N. A., Rachinger, W., Kunz, M., Thon, N., Flatz, W. H., Tonn, J. C. & Schichor, C. (2016) 'Orbit-associated tumors: navigation and control of resection using intraoperative computed tomography'. *J Neurosurg*, 124 (5), pp. 1319-1327.

Tetreault MP, Y. Y., Katz JP. (2013) 'Krüppel-like factors in cancer.'. *Nat Rev Cancer*, 13 (10), pp. 701-713.

Thery, C., Amigorena, S., Raposo, G. & Clayton, A. (2006) 'Isolation and characterization of exosomes from cell culture supernatants and biological fluids'. *Curr Protoc Cell Biol*, Chapter 3 pp. Unit 3 22.

Thery, C., Zitvogel, L. & Amigorena, S. (2002) 'Exosomes: composition, biogenesis and function'. *Nat Rev Immunol*, 2 (8), pp. 569-579.

Tian, T., Zhu, Y. L., Hu, F. H., Wang, Y. Y., Huang, N. P. & Xiao, Z. D. (2013) 'Dynamics of exosome internalization and trafficking'. *J Cell Physiol*, 228 (7), pp. 1487-1495.

Tiwari, P. C. & Pal, R. (2017) 'The potential role of neuroinflammation and transcription factors in Parkinson disease'. *Dialogues Clin Neurosci*, 19 (1), pp. 71-80.

Tominaga, N., Hagiwara, K., Kosaka, N., Honma, K., Nakagama, H. & Ochiya, T. (2014) 'RPN2-mediated glycosylation of tetraspanin CD63 regulates breast cancer cell malignancy'. *Mol Cancer*, 13 pp. 134.

Toulmonde, M., Blay, J. Y., Bouche, O., Mir, O., Penel, N., Isambert, N., Duffaud, F., Bompas, E., Esnaud, T., Boidot, R., Geneste, D., Ghiringhelli, F., Lucchesi, C., Bellera, C. A., Le Loarer, F. & Italiano, A. (2019) 'Activity and Safety of Palbociclib in Patients with Advanced Gastrointestinal Stromal Tumors

Refractory to Imatinib and Sunitinib: A Biomarker-driven Phase II Study'. *Clin Cancer Res*, 25 (15), pp. 4611-4615.

Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K. & et al. (1993) 'A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor'. *Cell*, 72 (5), pp. 791-800.

Tufan, K., Dogulu, F., Kurt, G., Emmez, H., Ceviker, N. & Baykaner, M. K. (2005) 'Intracranial meningiomas of childhood and adolescence'. *Pediatr Neurosurg*, 41 (1), pp. 1-7.

Tukachinsky, H., Petrov, K., Watanabe, M. & Salic, A. (2016) 'Mechanism of inhibition of the tumor suppressor Patched by Sonic Hedgehog'. *Proc Natl Acad Sci U S A*, 113 (40), pp. E5866-E5875.

Tumber, A., Nuzzi, A., Hookway, E. S., Hatch, S. B., Velupillai, S., Johansson, C., Kawamura, A., Savitsky, P., Yapp, C., Szykowska, A., Wu, N., Bountra, C., Strain-Damerell, C., Burgess-Brown, N. A., Ruda, G. F., Fedorov, O., Munro, S., England, K. S., Nowak, R. P., Schofield, C. J., La Thangue, N. B., Pawlyn, C., Davies, F., Morgan, G., Athanasou, N., Muller, S., Oppermann, U. & Brennan, P. E. (2017) 'Potent and Selective KDM5 Inhibitor Stops Cellular Demethylation of H3K4me3 at Transcription Start Sites and Proliferation of MM1S Myeloma Cells'. *Cell Chem Biol*, 24 (3), pp. 371-380.

Turner, N. C., Liu, Y., Zhu, Z., Loi, S., Colleoni, M., Loibl, S., DeMichele, A., Harbeck, N., Andre, F., Bayar, M. A., Michiels, S., Zhang, Z., Giorgetti, C., Arnedos, M., Huang Bartlett, C. & Cristofanilli, M. (2019) 'Cyclin E1 Expression and Palbociclib Efficacy in Previously Treated Hormone Receptor-Positive Metastatic Breast Cancer'. *J Clin Oncol*, 37 (14), pp. 1169-1178.

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J. & Lotvall, J. O. (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells'. *Nat Cell Biol*, 9 (6), pp. 654-659.

Vasudevan, H. N., Braunstein, S. E., Phillips, J. J., Pekmezci, M., Tomlin, B. A., Wu, A., Reis, G. F., Magill, S. T., Zhang, J., Feng, F. Y., Nicholaides, T., Chang, S. M., Sneed, P. K., McDermott, M. W., Berger, M. S., Perry, A. & Raleigh, D. R. (2018) 'Comprehensive Molecular Profiling Identifies FOXM1 as a Key Transcription Factor for Meningioma Proliferation'. *Cell Rep*, 22 (13), pp. 3672-3683.

Vaubel, R. A., Chen, S. G., Raleigh, D. R., Link, M. J., Chicoine, M. R., Barani, I., Jenkins, S. M., Aleff, P. A., Rodriguez, F. J., Burger, P. C., Dahiya, S., Perry, A. & Giannini, C. (2016) 'Meningiomas With Rhabdoid Features Lacking Other Histologic Features of Malignancy: A Study of 44 Cases and Review of the Literature'. *J Neuropathol Exp Neurol*, 75 (1), pp. 44-52.

Virgone, C., Cecchetto, G., Ferrari, A., Bisogno, G., Donofrio, V., Boldrini, R., Collini, P., Dall'Igna, P. & Alaggio, R. (2012) 'GATA-4 and FOG-2 expression in

pediatric ovarian sex cord-stromal tumors replicates embryonal gonadal phenotype: results from the TREP project'. *PLoS One*, 7 (9), pp. e45914.

Vogelbaum, M. A., Leland Rogers, C., Linskey, M. A. & Mehta, M. P. (2010) 'Opportunities for clinical research in meningioma'. *J Neurooncol*, 99 (3), pp. 417-422.

Wakana, K., Akiyama, Y., Aso, T. & Yuasa, Y. (2006) 'Involvement of GATA-4/-5 transcription factors in ovarian carcinogenesis'. *Cancer Lett*, 241 (2), pp. 281-288.

Walf-Vorderwulbecke, V., Pearce, K., Brooks, T., Hubank, M., van den Heuvel-Eibrink, M. M., Zwaan, C. M., Adams, S., Edwards, D., Bartram, J., Samarasinghe, S., Ancliff, P., Khwaja, A., Goulden, N., Williams, G., de Boer, J. & Williams, O. (2018) 'Targeting acute myeloid leukemia by drug-induced c-MYB degradation'. *Leukemia*, 32 (4), pp. 882-889.

Wang, D., Lu, M., Miao, J., Li, T., Wang, E. & Cui, Q. (2009) 'Cepred: predicting the co-expression patterns of the human intronic microRNAs with their host genes'. *PLoS One*, 4 (2), pp. e4421.

Wang, J., Wang, Q., Cui, Y., Liu, Z. Y., Zhao, W., Wang, C. L., Dong, Y., Hou, L., Hu, G., Luo, C., Chen, J. & Lu, Y. (2012) 'Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells'. *J Neurooncol*, 106 (3), pp. 473-484.

Wang, M., Deng, X., Ying, Q., Jin, T., Li, M. & Liang, C. (2015) 'MicroRNA-224 targets ERG2 and contributes to malignant progressions of meningioma'. *Biochem Biophys Res Commun*, 460 (2), pp. 354-361.

Wang, T., Gilkes, D. M., Takano, N., Xiang, L., Luo, W., Bishop, C. J., Chaturvedi, P., Green, J. J. & Semenza, G. L. (2014a) 'Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis'. *Proc Natl Acad Sci U S A*, 111 (31), pp. E3234-3242.

Wang, Z., Zhang, J., Ye, M., Zhu, M., Zhang, B., Roy, M., Liu, J. & An, X. (2014b) 'Tumor suppressor role of protein 4.1B/DAL-1'. *Cell Mol Life Sci*, 71 (24), pp. 4815-4830.

Weber, R. G., Bostrom, J., Wolter, M., Baudis, M., Collins, V. P., Reifenberger, G. & Lichter, P. (1997) 'Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression'. *Proc Natl Acad Sci U S A*, 94 (26), pp. 14719-14724.

Wei, W., Zhang, W. Y., Bai, J. B., Zhang, H. X., Zhao, Y. Y., Li, X. Y. & Zhao, S. H. (2016) 'The NF-kappaB-modulated microRNAs miR-195 and miR-497 inhibit myoblast proliferation by targeting Igf1r, Insr and cyclin genes'. *J Cell Sci*, 129 (1), pp. 39-50.

Wei, Y., Wang, D., Jin, F., Bian, Z., Li, L., Liang, H., Li, M., Shi, L., Pan, C., Zhu, D., Chen, X., Hu, G., Liu, Y., Zhang, C. Y. & Zen, K. (2017) 'Pyruvate kinase

type M2 promotes tumour cell exosome release via phosphorylating synaptosome-associated protein 23'. *Nat Commun*, 8 pp. 14041.

Weller, M., Roth, P., Sahm, F., Burghardt, I., Schuknecht, B., Rushing, E. J., Regli, L., Lindemann, J. P. & von Deimling, A. (2017) 'Durable Control of Metastatic AKT1-Mutant WHO Grade 1 Meningothelial Meningioma by the AKT Inhibitor, AZD5363'. *J Natl Cancer Inst*, 109 (3), pp. 1-4.

Wen, P. Y., Quant, E., Drappatz, J., Beroukhim, R. & Norden, A. D. (2010) 'Medical therapies for meningiomas'. *J Neurooncol*, 99 (3), pp. 365-378.

Whittle, I. R., Smith, C., Navoo, P. & Collie, D. (2004) 'Meningiomas'. *Lancet*, 363 (9420), pp. 1535-1543.

Wicking, C., Smyth, I. & Bale, A. (1999) 'The hedgehog signalling pathway in tumorigenesis and development'. *Oncogene*, 18 (55), pp. 7844-7851.

Wiemels, J., Wrensch, M. & Claus, E. B. (2010) 'Epidemiology and etiology of meningioma'. *J Neurooncol*, 99 (3), pp. 307-314.

Willis, J., Smith, C., Ironside, J. W., Erridge, S., Whittle, I. R. & Everington, D. (2005) 'The accuracy of meningioma grading: a 10-year retrospective audit'. *Neuropathol Appl Neurobiol*, 31 (2), pp. 141-149.

Wu, A., Garcia, M. A., Magill, S. T., Chen, W., Vasudevan, H. N., Perry, A., Theodosopoulos, P. V., McDermott, M. W., Braunstein, S. E. & Raleigh, D. R.

(2018) 'Presenting Symptoms and Prognostic Factors for Symptomatic Outcomes Following Resection of Meningioma'. *World Neurosurg*, 111 pp. e149-e159.

Yamashita, S., Fujisawa, M., Kodama, K., Ishikawa, M. & Katagi, R. (2013) 'Use of preoperative 3D CT/MR fusion images and intraoperative CT to detect lesions that spread onto the brain surface.'. *Acta Neurochir Suppl.*,

Yan, C. & Higgins, P. J. (2013) 'Drugging the undruggable: transcription therapy for cancer'. *Biochim Biophys Acta*, 1835 (1), pp. 76-85.

Yang, Y., Li, Z., Yuan, H., Ji, W., Wang, K., Lu, T., Yu, Y., Zeng, Q., Li, F., Xia, W. & Lu, S. (2019) 'Reciprocal regulatory mechanism between miR-214-3p and FGFR1 in FGFR1-amplified lung cancer'. *Oncogenesis*, 8 (9), pp. 50.

Yesiloz, U., Kirches, E., Hartmann, C., Scholz, J., Kropf, S., Sahm, F., Nakamura, M. & Mawrin, C. (2017) 'Frequent AKT1E17K mutations in skull base meningiomas are associated with mTOR and ERK1/2 activation and reduced time to tumor recurrence'. *Neuro Oncol*, 19 (8), pp. 1088-1096.

Yi, C., McCarty, J. H., Troutman, S. A., Eckman, M. S., Bronson, R. T. & Kissil, J. L. (2005) 'Loss of the putative tumor suppressor band 4.1B/Dal1 gene is dispensable for normal development and does not predispose to cancer'. *Mol Cell Biol*, 25 (22), pp. 10052-10059.

Yin, Y., Hong, S., Yu, S., Huang, Y., Chen, S., Liu, Y., Zhang, Q., Li, Y. & Xiao, H. (2017) 'MiR-195 Inhibits Tumor Growth and Metastasis in Papillary Thyroid Carcinoma Cell Lines by Targeting CCND1 and FGF2'. *Int J Endocrinol*, 2017 pp. 6180425.

Yu, B., Gong, M., He, Z., Wang, Y. G., Millard, R. W., Ashraf, M. & Xu, M. (2013) 'Enhanced mesenchymal stem cell survival induced by GATA-4 overexpression is partially mediated by regulation of the miR-15 family.'. *Int J Biochem Cell Biol.*, 45 (12), pp. 2724-2735.

Yu, Q., Geng, Y. & Sicinski, P. (2001) 'Specific protection against breast cancers by cyclin D1 ablation'. *Nature*, 411 (6841), pp. 1017-1021.

Yuzawa, S., Nishihara, H. & Tanaka, S. (2016) 'Genetic landscape of meningioma'. *Brain Tumor Pathol*, 33 (4), pp. 237-247.

Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J. & Mi, S. (2015) 'Exosome and exosomal microRNA: trafficking, sorting, and function'. *Genomics Proteomics Bioinformatics*, 13 (1), pp. 17-24.

Zhi, F., Shao, N., Li, B., Xue, L., Deng, D., Xu, Y., Lan, Q., Peng, Y. & Yang, Y. (2016) 'A serum 6-miRNA panel as a novel non-invasive biomarker for meningioma'. *Sci Rep*, 6 pp. 32067.

Zhi, F., Zhou, G., Wang, S., Shi, Y., Peng, Y., Shao, N., Guan, W., Qu, H., Zhang, Y., Wang, Q., Yang, C., Wang, R., Wu, S., Xia, X. & Yang, Y. (2013) 'A

microRNA expression signature predicts meningioma recurrence'. *Int J Cancer*, 132 (1), pp. 128-136.

Zhou, B., Li, H., Xing, C., Ye, H., Feng, J., Wu, J., Lu, Z., Fang, J. & Gao, S. (2017) 'Honokiol induces proteasomal degradation of AML1-ETO oncoprotein via increasing ubiquitin conjugase UbcH8 expression in leukemia.'. *Biochem Pharmacol.*,

Zhou, L. & Hanemann, C. O. (2012) 'Merlin, a multi-suppressor from cell membrane to the nucleus'. *FEBS Lett*, 586 (10), pp. 1403-1408.

Zomer, A., Maynard, C., Verweij, F. J., Kamermans, A., Schafer, R., Beerling, E., Schiffelers, R. M., de Wit, E., Berenguer, J., Ellenbroek, S. I. J., Wurdinger, T., Pegtel, D. M. & van Rheenen, J. (2015) 'In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior'. *Cell*, 161 (5), pp. 1046-1057.