Copyright Statement

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.
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:


Word count of main body of thesis: 49634

Signed

Date
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

1 Introduction 1

1.1 Background of the study 1

1.2 Biomarkers in neuro-oncology 3

1.3 Meningioma: origin and classification 8

1.4 Clinical presentation 11

1.5 Incidence and risk factors 12

1.6 The genetic landscape of meningioma 14

1.6.1 NF2-associated meningiomas 16

1.6.2 Mutations associated with non-NF2 meningioma 18

1.6.3 Cytogenetic alterations associated with malignancy 23

1.7 Current treatment options for meningioma 24

1.7.1 Surgery for intracranial meningioma 25

1.7.2 Radiation treatment 28

1.7.3 Systemic treatments 28

1.7.3.1 Molecular therapy 29

1.7.3.2 Immunotherapy 33

1.8 Issues regarding meningioma classification 34

1.8.1 Identification of methylation classes for meningioma prognosis 35
1.8.2 Identification of Cyclin D1 as a potential biomarker for meningioma 38

1.8.3 microRNAs as potential meningioma biomarkers 41

1.8.3.1 miRNA biogenesis and function 41

1.8.3.2 miRNA studies in meningioma 43

1.9 Exosomes 46

1.9.1 Role of exosomal miRNAs in cancer 52

1.10 Project aims 54

2 Materials and methods 56

2.1 Cells and exosome biology 56

2.1.1 Cells and tissues 56

2.1.2 Tumour digestion and cell culture 57

2.1.2.1 Recovery of cryopreserved cells 59

2.1.2.2 Cell counting 59

2.1.3 Exosome harvesting 60

2.1.3.1 From cell culture medium 60

2.1.3.2 From blood serum 60

2.2 Target identification and validation 61

2.2.1 Lentivirus-mediated transduction 61

2.2.2 In vitro pharmacology 63

2.2.2.1 Cell viability assay 64
2.3 Protein analysis

2.3.1 Western blotting

2.3.1.1 Protein extraction and quantification

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

2.3.1.3 Protein transfer and immunoassay

2.3.2 Immunohistochemistry

2.4 Polymerase chain reaction (PCR)

2.4.1 miRNA candidates identification

2.4.2 RNA extraction

2.4.3 Real Time PCR: microRNA expression analysis

2.4.4 Real Time qPCR: gene expression analysis

2.4.5 ROC curve analysis

2.5 Statistical analysis

3 Results

3.1 Identification of a miRNA cluster downregulated in malignant meningioma

3.1.2 Cyclin D1 is overexpressed in malignant meningioma

3.1.3 Cyclin D1 is targeted by the miR-15 family

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

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

3.1.6 Discussion

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

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

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

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

3.2.4 Discussion

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

3.3.1 Investigation of GATA-4 in meningioma

3.3.1.1 GATA-4 is upregulated in malignant meningioma tumours

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

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

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

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

3.3.4 Discussion

3.3.4.1 GATA-4 is upregulated in malignant meningioma

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

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

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

3.4.1 NSC140905 decreases malignant meningioma cell viability through upregulation of the miR-497~195 cluster
3.4.2 NSC140905 shows potential to synergize with PD0332991 in decreasing viability of malignant meningioma cells

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

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

3.4.2.3 Investigation of possible synergistic effects between NSC140905 and PD0332991

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

3.4.3 Discussion

4 Discussion

4.1 Introduction

4.2 Discovery of novel biomarkers for meningioma

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

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

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

4.4 GATA-4 as a potential therapeutic target for malignant meningioma
# Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Caption</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Biomarkers along the clinical continuum.</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic illustration of the meninges.</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>Histological variants of meningioma classification.</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic of genetic/cytogenetic alterations in meningioma.</td>
<td>16</td>
</tr>
<tr>
<td>1.5</td>
<td>Overview of activated signalling pathways and drug targets in meningioma.</td>
<td>29</td>
</tr>
<tr>
<td>1.6</td>
<td>Unsupervised clustering of 497 meningioma samples considered in the study.</td>
<td>36</td>
</tr>
<tr>
<td>1.7</td>
<td>Overview of the six methylation classes and their molecular and clinical characteristics.</td>
<td>37</td>
</tr>
<tr>
<td>1.8</td>
<td>Roles of Cyclins and Cyclin-dependent kinases in the cell division cycle.</td>
<td>39</td>
</tr>
<tr>
<td>1.9</td>
<td>miRNA maturation and RISC assembly.</td>
<td>42</td>
</tr>
<tr>
<td>1.10</td>
<td>Schematic representation of exosome and microvesicle biogenesis pathways.</td>
<td>47</td>
</tr>
<tr>
<td>1.11</td>
<td>Function of EVs in tumour-to-stroma, stroma-to-tumour and tumour-to-tumour communication, fuelling cancer progression.</td>
<td>51</td>
</tr>
<tr>
<td>3.1</td>
<td>Investigation of Cyclin expression levels in meningioma by Western blot and gene expression analysis.</td>
<td>82</td>
</tr>
<tr>
<td>3.2</td>
<td>CDK4 is overexpressed in KT21-MG1 cells.</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 3.3 Gene expression analysis performed by qPCR of the miR-15 family in meningioma tissues.

Figure 3.4 ROC curve analysis of the 2-miRNA signature.

Figure 3.5 Gene expression analysis performed by RT-qPCR of the miR-15 family in WHO I and II PDMN cells.

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.

Figure 3.7 Representative Western blots showing assessment of exosome enrichments.

Figure 3.8 Analysis of miRNA cargo in exosomes isolated from cell culture media.

Figure 3.9 Analysis of miRNA cargo in exosomes isolated from meningioma patients' serum samples.

Figure 3.10 ROC curve analysis of the 2-miRNA signature in circulating exosomes.

Figure 3.11 GATA-4 is upregulated in malignant meningioma tumour samples.

Figure 3.12 Gene expression and Western blot analysis reveal GATA-4 upregulation in KT21-MG1 compared to WHO I PDMN cells.

Figure 3.13 GATA-4 is overexpressed in KT21-MG1 compared to Ben-Men-1.

Figure 3.14 FOG2, GATA-6 and GATA-5 expression levels in Ben-Men-1 and KT21-MG1 cells.
Figure 3.15 Effects of lentiviral-mediated GATA-4 transduction in Ben-Men-1 cells. 121

Figure 3.16 Effects of lentiviral-mediated interference of GATA-4 in malignant KT21-MG1 cells. 124

Figure 3.17 Effects of lentiviral-mediated miR-195 transduction in KT21-MG1 cells. 127

Figure 3.18 NSC140905 chemical structure. 138

Figure 3.19 NSC140905 titration on KT21-MG1 cells. 139

Figure 3.20 Schwann cells positivity for S100. 140

Figure 3.21 Effects of NSC140905 on KT21-MG1 cell viability and miR-497~195 cluster expression. 142

Figure 3.22 KT21-MG1 cells viability following treatment with PD0332991 for 24 hours. 145

Figure 3.23 Effect of NSC140905 pre-treatment on KT21-MG1 cells. 147

Figure 3.24 Normalised isobologram for NSC140905 + PD0332991 combination. 150

Figure 3.25 Representative Western blot panels of the effects of treatment with the synergistic combinations of PD0332991 and NSC140905. 152
Table of Tables

Table 1.1 Simpson grades of resection and corresponding EORTC/RTOG definitions of extent of resection. 26

Table 2.1 Stacking gel recipe. 66

Table 2.2 Resolving gel recipe. 67

Table 2.3 Buffer recipes. 67

Table 2.4 Primary antibodies. 69

Table 2.5 Secondary antibodies. 70

Table 3.1 miR-15 family members. 85

Table 3.2 miR-15 family members bind to the 3'UTR regions of Cyclins mRNAs. 86

Table 3.3 Single IHC score values for GATA-4 intensity. 114

Table 3.4 miR-15 family binding sites on the 3' UTR of GATA-4 mRNA. 126

Table 3.5 Data entered in CompuSyn. 149
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular toxicity</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute</td>
</tr>
<tr>
<td>AKT1/3</td>
<td>AKT serine/threonine kinase 1/3</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans-retinoic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCND1-2-3, E1</td>
<td>Cyclin D1-2-3, E1</td>
</tr>
<tr>
<td>CD9</td>
<td>Cluster of differentiation 9</td>
</tr>
<tr>
<td>CD63</td>
<td>Cluster of differentiation 63</td>
</tr>
<tr>
<td>CDK2/4/6</td>
<td>Cyclin-dependent kinase 2/4/6</td>
</tr>
<tr>
<td>CDKN2A/B</td>
<td>Cyclin dependent kinase inhibitor 2A/B</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>Computerised tomography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DAL-1</td>
<td>Differentially expressed in adenocarcinoma of the lung</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DOTATATE</td>
<td>DOTA-(Tyr&lt;sup&gt;3&lt;/sup&gt;)-octreotate</td>
</tr>
<tr>
<td>DPI</td>
<td>Dots per inch</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemical luminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFR&lt;sup&gt;vIII&lt;/sup&gt;</td>
<td>Epidermal growth factor receptor variant III</td>
</tr>
<tr>
<td>EORTC</td>
<td>European organization for research and treatment of cancer</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETS</td>
<td>E-twenty-six</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FOG2</td>
<td>Friend of GATA 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA-4/5/6</td>
<td>GATA-Binding protein 4/5/6</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFM</td>
<td>Growth factor medium</td>
</tr>
<tr>
<td>GM130</td>
<td>Golgi matrix protein</td>
</tr>
<tr>
<td>GTR</td>
<td>Gross total resection</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HH</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HMC</td>
<td>Human meningeal cells</td>
</tr>
<tr>
<td>HPF</td>
<td>High power field</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>H-RAS</td>
<td>Harvey rat sarcoma viral oncoprotein</td>
</tr>
<tr>
<td><strong>H-RAS</strong>&lt;sup&gt;V12&lt;/sup&gt;</td>
<td>H-RAS variant with Valine at position 12 instead of Glycine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxiurea</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>KLF4</td>
<td>Krüppel-like factor 4</td>
</tr>
<tr>
<td>LA</td>
<td>Laminin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozigosity</td>
</tr>
<tr>
<td>MEKK3</td>
<td>Mitogen-activated protein/ERK kinase kinase 3</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia type 1</td>
</tr>
<tr>
<td>Merlin</td>
<td>Moesin Ezrin Redixin like protein</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MN</td>
<td>Meningioma</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MOT</td>
<td>Molecular target identification</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1/2</td>
<td>Mammalian target of rapamycin complex 1/2</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofibromatosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NMT</td>
<td>Normal meningeal tissue</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>Pasha</td>
<td>Partner of Drosha</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase-chain reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand 1</td>
</tr>
<tr>
<td>PDMN</td>
<td>Patient-derived meningioma cells</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PFS6</td>
<td>Progression free survival at 6 months</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PI3KCA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha</td>
</tr>
<tr>
<td>PLL</td>
<td>Polylysine</td>
</tr>
<tr>
<td>POLR2A</td>
<td>RNA polymerase II subunit A</td>
</tr>
<tr>
<td>PPIi</td>
<td>Protein-protein interaction inhibitors</td>
</tr>
<tr>
<td>Pre-miRNA</td>
<td>precursor miRNA</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>primary miRNA</td>
</tr>
<tr>
<td>PTCH1</td>
<td>Protein patched homolog 1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>pRB</td>
<td>Phosphorylated retinoblastoma protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation buffer</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RLC</td>
<td>RISC loading complex</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Radiation therapy</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SMARCB1</td>
<td>SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1</td>
</tr>
<tr>
<td>SMARCE1</td>
<td>SWI/SNF related matrix-associated actin-dependent regulator of chromatin subfamily E member 1</td>
</tr>
<tr>
<td>SMO</td>
<td>Signalling member smoothened</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>snRNA</td>
<td>Small nuclear RNA</td>
</tr>
<tr>
<td>SRC</td>
<td>Proto-oncogene Tyrosine-protein kinase SRC</td>
</tr>
<tr>
<td>SRS</td>
<td>Stereotactic radiosurgery</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor type IIA</td>
</tr>
<tr>
<td>STR</td>
<td>Subtotal resection</td>
</tr>
<tr>
<td>SUFU</td>
<td>Suppressor of fused homolog</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose Non-Fermentable</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAF7</td>
<td>TNF receptor-associated factor 7</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
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 lifetime 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 meningioma 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) inhibitors 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 activation and promotes proliferation and tumour growth (Carpten et al., 2007; Beaver et al., 2013).

PIK3CA

The PIK3CA gene encodes for the catalytic 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 heterozygosity 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\textsuperscript{INK4a}/p14\textsuperscript{ARF}), and CDKN2B (p15\textsuperscript{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 grades of resection and corresponding EORTC/RTOG definitions of extent of resection

<table>
<thead>
<tr>
<th>Simpson grade</th>
<th>Definition</th>
<th>Extent of resection</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Gross total resection of tumour, dural attachment, and abnormal bone</td>
<td>GTR</td>
</tr>
<tr>
<td>II</td>
<td>Gross total resection of tumour, coagulation of dural attachment</td>
<td>GTR</td>
</tr>
<tr>
<td>III</td>
<td>Gross total resection of tumour without resection or coagulation of dural</td>
<td></td>
</tr>
<tr>
<td></td>
<td>attachments, or extradural extensions</td>
<td>GTR</td>
</tr>
<tr>
<td>IV</td>
<td>Partial tumour resection</td>
<td>STR</td>
</tr>
<tr>
<td>V</td>
<td>Tumour biopsy</td>
<td>STR</td>
</tr>
</tbody>
</table>

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 trial 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 G1 phase of the cell cycle. In late G1, 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 G0/G1 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. 1.1). 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/mL penicillin/streptomycin (Gibco, Life Technologies, Loughborough, UK), and incubated at 37 °C in a humidified atmosphere (5% CO₂) 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% CO₂, 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% CO₂, 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% CO₂.

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 x 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:

\[
\text{MOI} = \frac{\text{Virus titre (IU/mL) x Virus volume (mL)}}{\text{Total cell number}}
\]

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.

<table>
<thead>
<tr>
<th>10 mL Stacking Gel</th>
<th>4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protogel 40% (BioRad, Watford, UK)</td>
<td>1 mL</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, 0.4% SDS, pH 6.8</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>milliQ water</td>
<td>6.38 mL</td>
</tr>
<tr>
<td>10% APS (Sigma Aldrich, Gillingham, UK)</td>
<td>100 µL</td>
</tr>
<tr>
<td>TEMED (ThermoFisher Scientific, Loughborough, UK)</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Table 2.1 Stacking gel recipe.
<table>
<thead>
<tr>
<th>20 mL Resolving gel</th>
<th>8% (25-200 kDa)</th>
<th>10% (15-100 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProtoGel 40% (BioRad, Watford, UK)</td>
<td>4 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>5 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>10% SDS (Fisher Scientific, Loughborough, UK)</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>milliQ water</td>
<td>10.58 mL</td>
<td>9.58 mL</td>
</tr>
<tr>
<td>10% APS (Sigma Aldrich, Gillingham, UK)</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>TEMED (ThermoFisher Scientific, Loughborough, UK)</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

Table 2.2 Resolving gel recipe.

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

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.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Species</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1 (92G2)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>2978T</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyclin D2 (D52F9)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>3741T</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyclin D3 (DCS22)</td>
<td>CST, London, UK</td>
<td>Mouse</td>
<td>2936T</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyclin E1 (HE12)</td>
<td>CST, London, UK</td>
<td>Mouse</td>
<td>4129T</td>
<td>1:1000</td>
</tr>
<tr>
<td>CDK2 (78B2)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>2546T</td>
<td>1:1000</td>
</tr>
<tr>
<td>CDK4 (D9G3E)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>12790T</td>
<td>1:1000</td>
</tr>
<tr>
<td>CDK6 (D4S8S)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>13331T</td>
<td>1:1000</td>
</tr>
<tr>
<td>GATA-4 (D3A3M)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>36966S</td>
<td>1:500</td>
</tr>
<tr>
<td>GATA-5</td>
<td>Abcam, Cambridge, UK</td>
<td>Rabbit</td>
<td>ab48820</td>
<td>1:500</td>
</tr>
<tr>
<td>GATA-6 (D61E4)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>5851T</td>
<td>1:500</td>
</tr>
<tr>
<td>Antibody</td>
<td>Manufacturer</td>
<td>Species</td>
<td>Catalog Number</td>
<td>Dilution</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------------</td>
<td>---------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>FOG-2</td>
<td>Abcam, Cambridge, UK</td>
<td>Rabbit</td>
<td>ab123879</td>
<td>1:500</td>
</tr>
<tr>
<td>Bcl-2 (124)</td>
<td>CST, London, UK</td>
<td>Mouse</td>
<td>15071T</td>
<td>1:1000</td>
</tr>
<tr>
<td>RB [EPR17512]</td>
<td>Abcam, Cambridge, UK</td>
<td>Rabbit</td>
<td>ab181616</td>
<td>1:500</td>
</tr>
<tr>
<td>pRB (Ser807/811) (D20B12) XP®</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>8516S</td>
<td>1:500</td>
</tr>
<tr>
<td>CD9 (D8O1A)</td>
<td>CST, London, UK</td>
<td>Rabbit</td>
<td>13174S</td>
<td>1:500</td>
</tr>
<tr>
<td>CD63 (MX-49.129.5)</td>
<td>Santa Cruz Biotechnology Inc., Heidelberg, Germany</td>
<td>Mouse</td>
<td>sc-5275</td>
<td>1:500</td>
</tr>
<tr>
<td>GM130 (35/GM130)</td>
<td>Becton Dickinson U.K. Ltd., Swindon, UK</td>
<td>Mouse</td>
<td>610823</td>
<td>1:500</td>
</tr>
<tr>
<td>Calnexin (H-70)</td>
<td>Santa Cruz Biotechnology Inc., Heidelberg, Germany</td>
<td>Rabbit</td>
<td>sc-11397</td>
<td>1:1000</td>
</tr>
<tr>
<td>GAPDH (6C5)</td>
<td>Millipore, Watford, UK</td>
<td>Mouse</td>
<td>MAB374</td>
<td>1:50000</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Antibody Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Rabbit HRP conjugated</td>
<td>BioRad, Watford, UK</td>
<td>170-6516</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-Mouse HRP conjugated</td>
<td>BioRad, Watford, UK</td>
<td>172-1019</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:5000</td>
</tr>
</tbody>
</table>

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 H₂O) 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 H₂O) 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-slippping 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


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

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

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 $2^{-(ΔΔCt)}$ (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

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 Log2 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 Log10 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).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Location</th>
<th>Name and sequence of mature miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a~16-1</td>
<td>13q14.3</td>
<td>miR-15a-5p 5’ 14 – uagccacauaaugguugug – 35 3’&lt;br&gt;miR-16-1-5p 5’ 14 – uagccacguaaaauuggcg – 35 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-15b-5p 5’ 20 – uagccacaucaugguuuaca – 41 3’&lt;br&gt;miR-16-2-5p 5’ 10 – uagccacguaaaauuggcg – 31 3’</td>
</tr>
</tbody>
</table>

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

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](image)

**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\textsubscript{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 G₀/G₁ checkpoint), and E1 (G₁/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 G₁ 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 ΔΔC_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 Log₁₀ fold increase), while showing a trend to increase in WHO II samples compared to WHO I (2.00 Log₁₀ 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\textsubscript{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).

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

**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.

![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).](image)

**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.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Position in the UTR</th>
<th>seed match</th>
<th>context++ score</th>
<th>context++ score percentile</th>
<th>weighted context++ score</th>
<th>conserved branch length</th>
<th>Pct</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-15a-5p</td>
<td>856-862</td>
<td>7mer-m8</td>
<td>-0.19</td>
<td>86</td>
<td>-0.19</td>
<td>3.194</td>
<td>0.5</td>
</tr>
<tr>
<td>hsa-miR-497-5p</td>
<td>856-862</td>
<td>7mer-m8</td>
<td>-0.18</td>
<td>86</td>
<td>-0.18</td>
<td>3.194</td>
<td>0.5</td>
</tr>
<tr>
<td>hsa-miR-15b-5p</td>
<td>856-862</td>
<td>7mer-m8</td>
<td>-0.18</td>
<td>85</td>
<td>-0.18</td>
<td>3.194</td>
<td>0.5</td>
</tr>
<tr>
<td>hsa-miR-195-5p</td>
<td>856-862</td>
<td>7mer-m8</td>
<td>-0.18</td>
<td>85</td>
<td>-0.18</td>
<td>3.194</td>
<td>0.5</td>
</tr>
<tr>
<td>hsa-miR-16-5p</td>
<td>856-862</td>
<td>7mer-m8</td>
<td>-0.17</td>
<td>84</td>
<td>-0.17</td>
<td>3.194</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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.

![NSC140905 chemical structure](image)

*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).](image)
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: G0 (quiescence), followed by G1 (pre-DNA synthesis), S (DNA synthesis), G2 (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.](image-url)
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 3.2.3 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<0.005).

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).
<table>
<thead>
<tr>
<th>Point #</th>
<th>Dose PD0332991</th>
<th>Dose NSC140905</th>
<th>Effect (Mortality)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.25 µM</td>
<td>50 µM</td>
<td>0.08</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>1.25 µM</td>
<td>100 µM</td>
<td>0.17</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>2.5 µM</td>
<td>50 µM</td>
<td>0.12</td>
<td>1.28</td>
</tr>
<tr>
<td>4</td>
<td>2.5 µM</td>
<td>100 µM</td>
<td>0.2</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>5 µM</td>
<td>50 µM</td>
<td>0.04</td>
<td>18.34</td>
</tr>
<tr>
<td>6</td>
<td>5 µM</td>
<td>100 µM</td>
<td>0.19</td>
<td>0.83</td>
</tr>
<tr>
<td>7</td>
<td>10 µM</td>
<td>50 µM</td>
<td>0.16</td>
<td>1.06</td>
</tr>
<tr>
<td>8</td>
<td>10 µM</td>
<td>100 µM</td>
<td>0.34</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**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


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


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.,


including NEK9, HK2 and SET and deregulation of RNA metabolism'.

_EBioMedicine_, 40 pp. 77-91.


time of resistance to palbociclib in hormone receptor-positive breast cancer'.

*NPJ Breast Cancer*, 4 pp. 38.


resistance to the CDK4/6 specific inhibitor palbociclib in gastric cancer cells'.


Perry, A., Giannini, C., Raghavan, R., Scheithauer, B. W., Banerjee, R.,
(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.


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

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'.


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.,


Porta, C., Paglino, C. & Mosca, A. (2014) 'Targeting PI3K/Akt/mTOR Signaling in Cancer'. *Front Oncol*, 4 pp. 64.


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


pediatric ovarian sex cord-stromal tumors replicates embryonal gonadal phenotype: results from the TREP project'. *PLoS One*, 7 (9), pp. e45914.


