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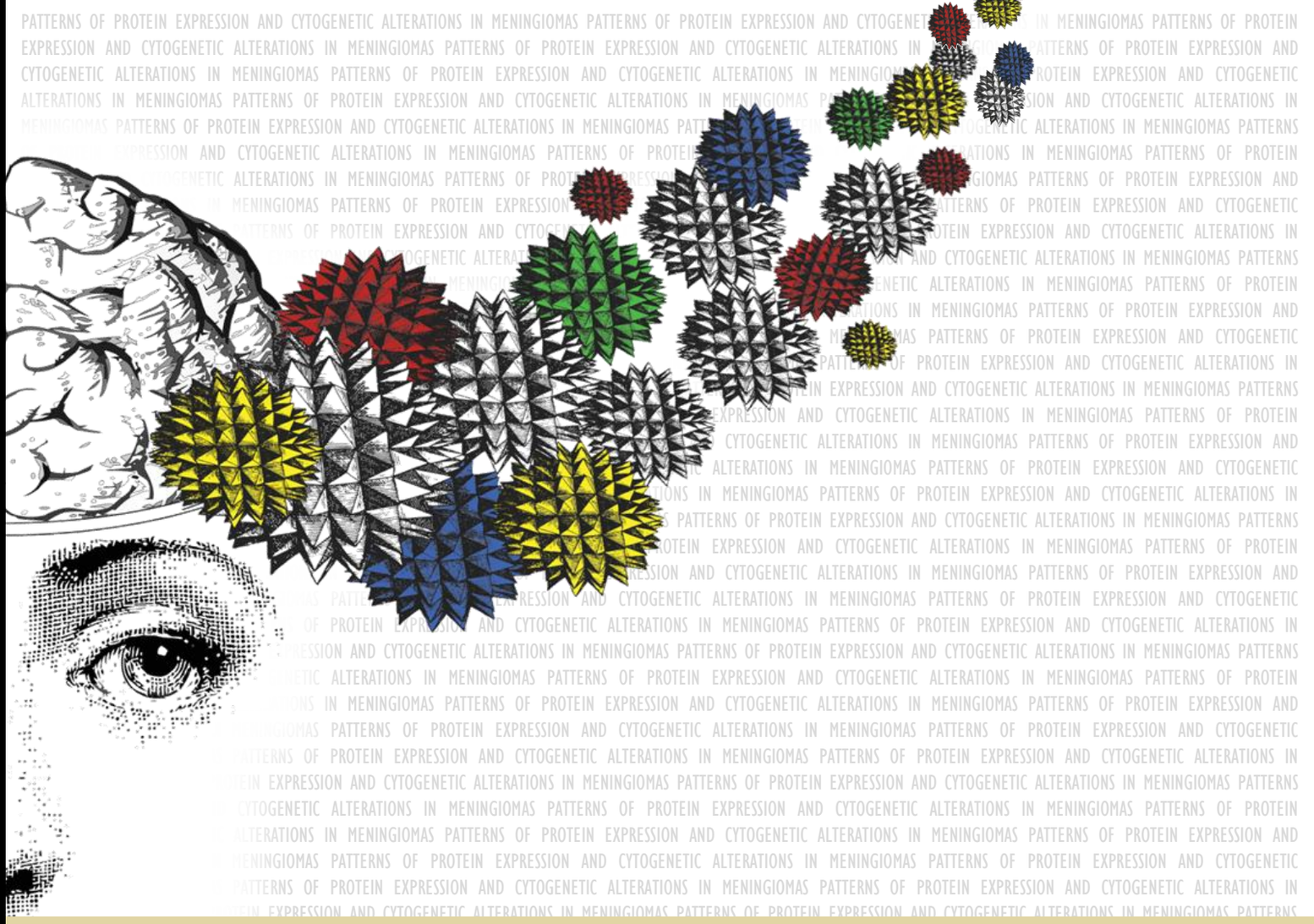
PATTERNS OF PROTEIN EXPRESSION AND CYTOGENETIC ALTERATIONS IN MENINGIOMAS: RELATIONSHIP WITH THE CLINICAL AND BIOLOGICAL FEATURES OF THE DISEASE

UNIVERSIDADE DE COIMBRA



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Tese de Doutoramento em Ciências Farmacêuticas, especialidade de Biologia Celular e Molecular,
orientada por Alberto Orfão e Maria Celeste Lopes e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Universidade de Coimbra

Patterns of protein expression and cytogenetic alterations in meningiomas: relationship with the clinical and biological features of the disease

Perfis de expressão proteica e alterações genéticas em meningiomas: relação com as características clinicas e biológicas da doença

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Tese de Doutoramento em Ciências Farmacêuticas, na especialidade de Biologia Celular e Molecular, apresentada à Faculdade de Farmácia da Universidade de Coimbra
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Table of contents

Abbreviations

A

ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABL	c-abl oncogene 1, non-receptor tyrosine kinase
ADSL	Adenylosuccinate lyase
Ag	Antigen
AKT1	v-akt murine thymoma viral oncogene homolog 1, serine/threonine protein kinase B (also known as AKT, PKB)
ALCAM	Activated leukocyte adhesion molecule (also known as CD166)
ALDOA	Aldolase A, fructose-bisphosphate
ALPL	Alkaline phosphatase
APC (cell)	Antigen presenting cell
APC (gene)	Adenomatous polyposis coli
APM-1	Adipocyte-specific secretory protein
Arg1	Arginase 1

B

B7-H1	Programmed cell death 1 (also known as PDCD1)
BAM22	Adaptor-related protein complex 1, beta 1 subunit (also known as AP1B1)
BBB	Blood-brain barrier
BCL2	B-cell lymphoma 2
BCR	Breakpoint cluster region
BID	BH3 interacting domain death agonist
BMP	Bone morphogenic proteins
BMPR	Bone morphogenic protein receptor

C

C	Complement component
CBTRUS	Central Brain Tumor Registry of the United States
CCL	Chemokine (C-C motif) ligand
CCND1	Cyclin D1
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CD2	Lymphocyte function-associated antigen 2 (LFA-2)
CD3	CD3 molecule (T cell marker)
CD4	CD4 molecule (TCD4 cell marker)
CD8	CD8 molecule (TCD8 cell marker)
CD9	Tetraspanin-29 (TSPAN29)
CD11b	Integrin, alpha M (ITGAM, also known as CR3A - complement component 3 receptor 3 subunit)
CD11c	Integrin, alpha X (ITGAX, also known as complement component 3 receptor 4 subunit)
CD13	Alanyl aminopeptidase (also known as ANPEP, APN)
CD14	CD14 molecule
CD16	Fc fragment of IgG, low affinity III receptor (FCGR3A)
CD19	CD19 molecule (B-lymphocyte antigen)
CD22	CD22 molecule (SIGLEC2)
CD25	Interleukin 2 receptor, alfa (IL2RA)
CD28	CD28 molecule
CD32	Fc fragment of IgG, low affinity II receptor (FCGR2A)

CD33	CD33 molecule (SIGLEC3)
CD37	Tetraspanin-26 (TSPAN26)
CD38	Cyclic ADP ribose hydrolase
CD40	TNF receptor superfamily member 5
CD45	Leukocyte common antigen (also known as PTPRC - protein tyrosine phosphatase, receptor type, C)
CD53	Tetraspanin-25 (TSPAN25)
CD55	Decay accelerating factor for complement (DAF)
CD56	Neural cell adhesion molecule 1 (NCAM1)
CD57	Beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)(B3GAT1)
CD58	Lymphocyte function-associated antigen 3 (LFA-3)
CD59	Complement regulatory protein, protectin
CD63	Tetraspanin-30 (TSPAN30)
CD64	Fc fragment of IgG, low affinity I receptor (FCGR1A)
CD68	CD68 molecule
CD69	CD69 molecule
CD74	Major histocompatibility complex, class II (HLA-DG)
CD80	CD80 molecule (also known as B7.1)
CD81	Tetraspanin-28 (TSPAN28)
CD86	CD86 molecule (also known as B7.2)
CD95	Fas cell surface death receptor (FAS)
CD99	CD99 molecule
CD127	Interleukin 7 receptor (IL7R)
CD133	Prominin 1 (PROM1)
CD163	CD163 molecule, scavenger receptor
CD200	OX-2 membrane glycoprotein
CD204	Macrophage scavenger receptor 1 (MSR1)
CD206	Mannose receptor, C type 1 (MRC1)
CDH1	Cadherin 1, type 1, E-cadherin
CDK4	Cyclin-dependent kinase 4
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A (also known as p16, p16INK4A, ARF, INK4A)
CDKN2B	Cyclin-dependent kinase inhibitor 2B (also known as p15, p15INK4B, INK4B)
CDKN2C	Cyclin-dependent kinase inhibitor 2C (also known as p18)
cDNA	complementary DNA
CEP7	Centromeric probe
CGH	Comparative genomic hybridization
CHKB	Choline kinase beta
CI	Confidence interval
CLEC7A	C-type lectin domain family 7, member A
CN	Copy number
CNS	Central nervous system
CNV	Copy number variation
COL8A2	Collagen, type VIII, alpha 2
COX	Cyclooxygenase
CR	Complement component receptor
CRL4	Cullin-ring E3 ligase 4
CSF	Colony stimulating factor
CSF	Cerebrospinal fluid

CSFR	Colony stimulating factor receptor
CT	Computerized tomography
CTGF	Connective tissue growth factor
CTNNB1	Catenin beta 1
CXC	CXC chemokine
CXCR	CXC chemokines receptor
Cy	Cytoplasmic
CYBB	Cytochrome b-245, beta polypeptide

D

DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DAL-1	Erythrocyte membrane protein band 4.1-like 3 (also known as EPB41L3, 4.1B)
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cell
DCC	Deleted in colorectal carcinoma
DEFB1	Defensin, beta 1
del	deletion
DEPC	Diethylpyrocarbonate
DLL1	Delta-like 1
DMBT1	Deleted in malignant brain tumors 1
DNA	Deoxyribonucleic acid
DRAQ5	1,5-bis{[2-(di-methylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9,10-dione

E

EDTA	Ethylenediaminetetraacetic acid
EFS	Embryonal Fyn-associated substrate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELN	Elastin
ENC1	Ectodermal-neural cortex 1
EPB41	Erythrocyte membrane protein band 4.1
ERK	Extracellular signal-regulated kinase (also known as MAPK1)
ESR1	Estrogen receptor 1

F

F	Female
FACS	Fluorescence activated cell sorting
FAT3	FAT atypical cadherin 3
FBLN1	Fibulin 1
FBS	Fetal bovine serum
FcGR	Immunoglobulin Fc receptor
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (also known as CD64)
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (also known as CD32)
FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (also known as CD16b)
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FOXM1	Forkhead box M1
FOXP3	Forkhead box P3
FSC	Forward light scatter

G

GAB2	GRB2-associated binding protein 2
GADD45A	Growth arrest and DNA-damage-inducible, alpha
GBM	Glioblastoma multiforme
GEO	Gene Expression Omnibus
GEP	Gene expression profile
GFAP	Glial fibrillary acidic protein
GLI	GLI family zinc finger
GLIS	GLIS family zinc finger
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPS	Genetic progression score
GR	Glucocorticoid receptor
GSTM	Glutathione S-transferase mu
GSTP1	Glutathione S-transferase pi 1

H

HCK	Hemopoietic cell kinase
Her2/neu	Receptor tyrosine-protein kinase erbB-2, proto-oncogene Neu
HES	Hairy/Enhancer of Split
HGF	Hepatocyte growth factor
Hh	Hedgehog
hi	high
HIF-1	Hypoxia inducible factor-1 (also known as HIF1A)
HIST1H1C	Histone cluster 1, H1c (also known as H1.2)
HLA	Human leukocyte antigen
HLA-I	Human Leukocyte Antigen Class I
HLA-DR, DM, DQ	Human Leukocyte Antigen Class II
HOX	Homeobox
HPF	High-power magnification fields
HR	Hazard ratio
HRP	Horseradish peroxidase

I

iFISH	interphase Fluorescence in situ hybridization
IFN	Interferon
IGF	Insulin-like growth factor
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
IGFBP2	Insulin-like growth factor binding protein 2
IGFR	Insulin-like growth factor receptor
IgH	Immunoglobulin heavy locus

IHC	Immunohistochemistry
IL	Interleukin
INPP5D	Inositol polyphosphate-5-phosphatase
IP3	Inositol 1,4,5-triphosphate
IPA	Ingenuity Pathway Analysis
IRF	Interferon regulatory factor
ITG	Integrin

J

JAK	Janus kinase
JNK	c-Jun-NH2-kinase

K

KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1
KLF4	Kruppel-like factor 4 (gut)
KNS2	Kinesin light chain 1 (also known as KLC1)

L

LEPR	Leptin receptor
lo	low
LOH	Loss of heterozygosity
LPHN2	Latrophilin 2
LPS	Lipopolysaccharide
LSI	Locus specific identifier
LTBP	Latent transforming growth factor beta binding protein
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog

M

M	Male
mAb	Monoclonal antibody
MADH	SMAD family member (also known as SMAD)
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
MCP	Monocyte chemotactic protein
MDM2	Murine double minute 2 protein
MDSC	Myeloid-derived suppressor cell
MEG3	Maternally expressed gene 3
MEK	Mitogen-activated protein kinase kinase (also known as MAPK2)
MFC	Multiparameter flow cytometry
MFI	Mean fluorescence intensity
MGMT	O6-methylguanine–DNA methyltransferase
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MLK3	Mitogen-activated protein kinase kinase kinase 11 (also known as MAP3K11)
MLLT10	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10
MLPH	Melanophilin

MMP	Metalloproteinases
MR	Mannose receptor
MRI	Magnetic resonance imaging
mRNA	messenger Ribonucleic acid
mTOR	Mammalian target of Rapamycin
mTORC1	Mammalian target of Rapamycin complex 1
mTORC2	Mammalian target of Rapamycin complex 2
MXI1	MAX interactor 1, dimerization protein
MYD88	Myeloid differentiation primary response 88
MYO1F	Myosin IF
MYPT1	Myosin phosphatase targeting subunit 1

N

NDRG2	N-Myc downstream-regulated gene 2
NF2 (gene)	Neurofibromin 2
NF2 (disease)	Neurofibromatosis type 2
NF-κB	Nuclear factor κB
NG2	Neuron-glial antigen 2 (also known as CSPG4)
NK	Natural killer
NME1	NME/NM23 nucleoside diphosphate kinase 1
NOS2	Nitric oxide synthase 2 (also known as iNOS - inducible nitric oxide synthase)
NPLOC4	Nuclear protein localization 4 homolog
NR	Not reached
NS	Not significant (statistically)

P

PacB	Pacific blue
PacO	Pacific orange
PACS2	Phosphofurin acidic cluster sorting protein 2
PACSIN2	Protein kinase C and casein kinase substrate in neurons 2
PAK	p21-activated kinase
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor-beta
PE	Phycoerythrin
PEP	Protein expression profiles
PET	Positron Emission Tomography
PGE2	Prostaglandin E2
PI	Proliferation index
PI3K	Phosphinositide-3-kinase
PIK3CD, G	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta/gamma
PIP2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLA2	Phospholipase A2
PLCB1	Phospholipase C, beta 1
PLC-γ1	Phospholipase C, gamma 1
PML	Promyelocytic leukemia
PMM1	Phosphomannomutase 1
PR	Progesterone receptor
PRKCD	Protein kinase C, delta
PTCH1	Patched 1
PTEN	Phosphatase and tensin homolog
PTPN6	Protein tyrosine phosphatase, non-receptor type 6

R

Rac1	Ras-related C3 botulinum toxin substrate (rho family, small GTP binding protein Rac1)
RAR-α	Retinoic acid receptor, alpha
Ras	Rat sarcoma
RB1	Retinoblastoma 1 (also known as RB, pRb)
RFS	Relapse-free survival
RMA	Robust microarray normalization
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROC	Receiver operating characteristic
ROI	Reactive oxygen intermediates
RPMI	Roswell Park Memorial Institute medium
RPS6K	Ribosomal protein S6 kinase (also known as RPS6KB1, $p70^{S6K}$)
RT	Radiotherapy

S

SAM	Significance analysis of microarray
SD	Standard deviation
SDF1	Stromal cell derived factor 1
SEMA3A	Semaphorin 3A
SEMA4D	Semaphorin 4D
SFRP	Secreted frizzled-related proteins
SG	Spectrum green
SMO	Smoothened, frizzled class receptor
SNP	Single nucleotide polymorphism
SO	Spectrum orange
SPECT	Single photon emission computed tomography
SPP1	Secreted phosphoprotein 1
SR	Scavenger receptor
SRS	Stereotactic radiosurgery
SSC	Sideward light scatter
STAT3	Signal transducer and activator of transcription 3
SYK	Spleen tyrosine kinase

T

TAM	Tumor-infiltrating macrophages
TCN2	Transcobalamin II
TCR	T-cell receptor
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
Th	T helper
THBS1	Thrombospondin 1
TIL	Tumor-infiltrating lymphocytes
TiMa	Tissue macrophage
TIMP	Tissue inhibitors of metalloproteinases
TLE	Transducin-like enhancer of split
TLR	Toll like receptor
TMED9	Transmembrane emp24 protein transport domain containing 9
TMEM30B	Transmembrane protein 30B
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP53	Tumor protein p53
TP73	Tumor protein p73
TRAF7	TNF receptor-associated factor 7, E3 ubiquitin protein ligase
Treg	T regulatory cell
TREM2	Triggering receptor expressed on myeloid cells
TSLC1	Tumor suppressor in lung cancer 1 (also known as CADM1 - cell adhesion molecule 1)
TYROBP	TYRO protein tyrosine kinase binding protein

V

VCAM-1	Vascular cell adhesion molecule 1
VEGFA	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very Late Antigen-4 (Integrin alpha4beta1)

W

WAS	Wiskott-Aldrich syndrome
WHO	World Health Organization
WNK2	WNK lysine deficient protein kinase 2
WNT	Wingless

Z

ZFYVE21	Zinc finger, FYVE domain containing 21

Abstract/Resumo

ABSTRACT

Meningiomas are relatively common central nervous system tumors. Despite being mostly classified as benign/WHO grade I lesions, tumor recurrence still occurs in a significant proportion of cases. Thus, additional parameters are needed for better stratification and improved clinical management of meningioma patients, tumor cytogenetics and microenvironment-associated variables being of potential utility. In this regard, tumor cytogenetics has long emerged as a major source of biological variability in meningiomas and different cytogenetic profiles have been described (e.g. diploid tumors, tumors with isolated monosomy 22/del(22q) or with complex karyotypes) which are associated with patient outcome. In turn, tumor microenvironment has also been shown to be closely associated with tumor behavior due to the critical role that some infiltrating (immune) cells play in tumor growth; however, despite the presence of tumor-infiltrating immune cells in meningiomas has been recurrently reported, an association between such infiltrates and other features of the disease has yet not been demonstrated.

In this study, we investigated the cytogenetic and protein expression profiles of meningioma cells, as well as the immune cells infiltrating the tumor; our major goal focused on determining their potential association with other features of the disease, including outcome, in order to better understand the biology of meningiomas at both the tumor cell and the microenvironmental levels.

Firstly, we used multiparameter flow cytometry (MFC) immunophenotyping to analyze the overall cellular composition of 51 meningioma samples, as well as the immunophenotypic profile of the different major cell populations coexisting in the tumors, using a large panel of markers together with morphological, cytogenetic and phagocytic/endocytic analyses. Overall, coexistence of $CD45^-$ neoplastic cells and $CD45^+$ immune infiltrating cells was systematically detected among the meningioma samples. Infiltrating cells included a major population of tissue macrophages (TiMa), with an HLA-$DR^+CD14^+CD45^+CD68^+CD16^{-/+}CD33^{-/+}$ phenotype and high phagocytic/endocytic activity, together with cytotoxic lymphocytes (mostly T $CD8^+$- and NK-cells) at lower levels.

In the second part, we investigated the potential association between the number and immunophenotype of the different cell populations identified within the tumor samples and the clinico-biological features of the disease, including the cytogenetic patterns and gene expression profiles (GEP) of tumor cells, as evaluated by interphase fluorescence in situ hybridization (iFISH) and DNA-oligonucleotide arrays, respectively. Overall, a close association between the amount and cellular composition of the immune infiltrates and the cytogenetic

profile of the tumor, was found. Of note, meningiomas with isolated monosomy 22/del(22q) had greater numbers of TiMa (with a more activated and functionally mature phenotype), NK cells and (recently)-activated $CD69^+$ lymphocytes, together with a unique GEP characterized by an increased expression of genes involved in inflammatory/immune responses, which may contribute to partially explain the better outcome of this cytogenetic subgroup of meningiomas. Interestingly, although some meningiomas with complex karyotypes also showed high levels of TiMa infiltration, a polarization towards an M2-phenotype ($CD206^+$ cells) in association with infiltration by T regulatory cells was observed in these cases, which might contribute to explain the greater recurrence rate of this subgroup of tumors.

Similarly, the cytogenetic profile of meningiomas was also closely associated with the pattern of protein expression of tumor cells. Thus, diploid meningiomas displayed higher levels of expression of the CD55 complement regulatory protein, tumors carrying isolated monosomy 22/del(22q) showed greater levels of bcl2 and PDGFRβ and meningiomas carrying complex karyotypes displayed a greater proliferation index in association with decreased expression of the CD13 ectoenzyme, the CD9 and CD81 tetraspanins, and the Her2/neu growth factor receptor. Of note, some clinical features were also closely associated with specific immunophenotypic profiles (e.g. greater expression of CD53 and CD44 with a poorer outcome), in the absence of an independent prognostic value on relapse-free survival (RFS).

In the last part of this work, we aimed at building a prognostic scoring system for risk stratification of meningiomas based on a series of 302 patients followed for >5 years, in whom cytogenetic data was available. Multivariate analysis showed that age <55 years, tumor size >50mm, tumor localization at the intraventricular and anterior cranial base areas, WHO grade II/III and complex karyotypes were the only independent prognostic factors with an adverse impact on RFS; based on these five variables patients were stratified into four risk-categories with a significantly different ($p<0.001$) outcome, including both a good-prognosis and a very poor-prognosis subgroup of meningiomas (10 years RFS of 100%±0% and 0%±0%, respectively). Of note, the prognostic impact of the scoring system was retained when only WHO grade I cases were considered ($p<0.001$), being also validated in an independent series of 132 cases analyzed by 500K single nucleotide polymorphism-arrays.

In conclusion, here we propose a new MFC-based strategy to identify and characterize the different cell populations coexisting in meningiomas, and their patterns of protein expression, both parameters being closely associated with tumor cytogenetics. These results suggest the involvement of different signaling pathways in the distinct cytogenetic subgroups of meningiomas, at the same time they would contribute to explain the close association between tumor cytogenetics and patient outcome. In fact, the cytogenetic profile of

meningiomas proved to be critically relevant to predict patient RFS and thus, potentially also the most appropriate follow-up (and eventually treatment) strategy to be adopted for meningioma patients at different risk of relapse.

RESUMO

Os meningiomas são tumores relativamente comuns do sistema nervoso central. Apesar de serem classificados maioritariamente como benignos/lesões de grau I segundo a OMS, numa percentagem significativa de casos ainda ocorre recidiva após cirurgia. Deste modo, continua a ser necessário identificar parâmetros adicionais que permitam uma melhor estratificação de risco e ajudem no acompanhamento clínico dos doentes, estando entre os potencialmente mais informativos a citogenética e as variáveis associadas ao microambiente tumoral. Do ponto de vista citogenético, têm sido descritos vários perfis em meningiomas (p.e. tumores diplóides, com monossomia 22/del(22q) isolada ou cariótipos complexos), os quais estão associados ao prognóstico. Do mesmo modo, tem sido descrita uma estreita relação entre o microambiente e a evolução do tumor, uma vez que várias das células infiltrantes (p.e. do sistema imune) podem desempenhar importantes funções no controlo do crescimento tumoral. No entanto, apesar de já terem sido identificadas células do sistema imune no microambiente de meningiomas, a sua associação com outras características da doença, assim como o seu papel no desenvolvimento do tumor, é ainda pouco claro.

Neste estudo, propusemo-nos analisar os perfis citogenéticos e de expressão proteica das células de meningiomas e a caracterizar as células imunes infiltrantes do tumor; o nosso principal objetivo centrou-se na determinação da possível associação entre os perfis identificados e as outras características da doença, de forma a compreender melhor a biologia dos meningiomas a nível da célula tumoral e do seu microambiente.

Na primeira parte, utilizámos citometria de fluxo multiparamétrica (CFM) para determinar a composição celular de 51 amostras de meningiomas, assim como os perfis imunofenotípicos das várias populações de células coexistentes nos tumores, usando um amplo painel de marcadores juntamente com análises morfológicas, citogenéticas e funcionais. De forma geral, a coexistência de células neoplásicas $CD45^-$ e células imunes $CD45^+$ infiltrantes foi, sistematicamente, detetada em todas as amostras de meningioma estudadas. Entre as células infiltrantes estava incluída uma população maioritária de macrófagos (TiMa), com fenótipo HLA-$DR^+CD14^+CD45^+CD68^+CD16^{-/+}CD33^{-/+}$ e com elevada capacidade fagocítica/endocítica, junto com uma população minoritária de linfócitos citotóxicos (na sua maioria T $CD8^+$ e células NK).

Na segunda parte, investigámos a possível relação entre o número e imunofenótipo das diferentes populações de células identificadas nas amostras de meningiomas e as características clinico-biológicas da doença, incluindo os perfis citogenéticos e de expressão génica da célula tumoral, avaliados através de hibridização *in situ* fluorescente em núcleos

interfásicos (iFISH) e *microarrays* de oligonucleótidos de cDNA, respetivamente. Os resultados obtidos mostraram uma forte associação entre a quantidade e o tipo de células imunes e os perfis citogenéticos do tumor. É de referir que meningiomas com monossomia 22/del(22q) isolada apresentavam números mais elevados de TiMa (com um fenótipo mais ativado e funcionalmente maduro), células NK e linfócitos ($CD69^{+}$) ativados, com um perfil de expressão génica caracterizado pelo aumento da expressão de genes envolvidos em respostas inflamatórias/imunes; estes resultados permitem explicar o melhor prognóstico deste subgrupo citogenético de meningiomas. Curiosamente, parte dos meningiomas com cariótipo complexo também mostraram uma elevada infiltração de TiMa, no entanto, estes casos mostraram um fenótipo com polarização M2 ($CD206^{+}$), associado a um aumento na infiltração por parte de células T reguladoras, o que poderá ajudar a explicar as elevadas taxas de recidiva deste subgrupo citogenético de meningiomas.

Da mesma forma, os perfis citogenéticos dos meningiomas evidenciaram, também, uma forte associação com os perfis de expressão proteica das células tumorais. Assim, meningiomas diplóides apresentavam elevados níveis de expressão da proteína reguladora do complemento CD55, tumores com monossomia 22/del(22q) apresentavam níveis elevados das proteínas bcl2 e PDGFRβ, e meningiomas com cariótipos complexos mostravam uma maior taxa de proliferação associada a uma expressão reduzida dos níveis da ectoenzima CD13, das tetraspaninas CD9 e CD81 e do recetor do fator de crescimento Her2/neu. Além disto, algumas das características clínicas estavam, também, intimamente relacionadas com perfis imunofenotípicos específicos (p.e. expressão elevada de CD53 e CD44 e uma menor sobrevida livre de recidiva - SLR), apesar de não apresentarem um valor prognóstico independente.

Na última parte deste trabalho, procurámos construir um sistema de estratificação prognóstica de meningiomas com base numa série de 302 doentes seguidos durante >5 anos, nos quais os perfis citogenéticos estavam disponíveis. A análise multivariada mostrou que uma idade <55 anos, um tamanho do tumor >50mm, com localização nas áreas intraventriculares e na base (anterior) do crânio, histologia de grau II/III da OMS e cariótipos complexos eram os únicos fatores prognósticos independentes com um impacto negativo na SLR; com base nestes 5 parâmetros, os doentes foram classificados em quatro categorias de risco com prognósticos significativamente diferentes ($p<0.001$), incluindo subgrupos de bom-prognóstico e mau-prognóstico com taxas de SLR aos 10 anos de 100%±0% e 0%±0%, respetivamente. É importante notar que o impacto prognóstico deste sistema de estratificação se manteve ainda quando apenas se consideraram na análise os tumores de grau I OMS ($p<0.001$), sendo também validados numa série independente de 132 casos analisados por *single nucleotide polymorphism-arrays*.

Em conclusão, neste trabalho propomos uma nova estratégia com base em CFM para identificar e caracterizar as diferentes populações de células coexistentes em meningiomas e os seus perfis de expressão proteica, estando ambos os parâmetros intimamente relacionados com os perfis citogenéticos do tumor. Estes resultados sugerem o envolvimento de diferentes vias de sinalização nos diferentes subgrupos citogenéticos de meningiomas, ao mesmo tempo que permitem explicar a estreita relação entre a citogenética tumoral e o prognóstico dos doentes. De facto, os perfis citogenéticos de meningiomas demonstraram ter uma relevância crítica na previsão da evolução da doença e, consequentemente, na possível estratégia de monitorização (e tratamento) de doentes com meningioma com diferente risco de recidiva.

Chapter I – Introduction

1. CLINICAL HISTOPATHOLOGICAL AND EPIDEMIOLOGICAL FEATURES OF MENINGIOMAS

Meningiomas are one of the most frequent primary brain neoplasias, accounting for around 30-35% of all central nervous system (CNS) tumors [1, 2]. They originate from the meningeal coverings of the brain and the spinal cord [3]; due to the great cytological similarities between meningioma tumor cells and the arachnoidal cap cells, these latter cells most likely represent the normal counter part of the cell of origin of meningiomas. The normal arachnoidal cells form the outer layer of the arachnoid mater and the arachnoid villi, which are responsible for cerebrospinal fluid (CSF) drainage into the dural sinuses and veins [4, 5]. Meningiomas are histologically subdivided into different subtypes according to the World Health Organization (WHO) classification of CNS tumors. Despite the vast majority of meningiomas are considered to be benign and slow-growing neoplastic lesions, these tumors present with a great heterogeneity as regards symptoms, histopathology, recurrence rates, clinical aggressiveness, and outcome.

1.1. Incidence of meningiomas

Based on the most recent report of the Central Brain Tumor Registry of the United States (CBTRUS) for the years 2006-2010 [1], meningiomas show an incidence rate of 7.44 per 100.000 individuals. However, the incidence rate of meningiomas increases with age, since meningiomas are most commonly diagnosed in elderly patients, in between the sixth and seventh life decade [5, 6]. There is a clear female predominance with a more than two-fold higher incidence in females *vs.* males (age-adjusted incidence rate of 8-13 and 3-5 for 100.000 person/year for females and males, respectively) [1, 7]. The bias towards women is even greater in spinal meningiomas, where an imbalanced female/male ratio of 10:1 is observed. In contrast, more aggressive forms of meningioma show a predominance in males as well as in children [6].

1.2. Etiology and risk factors for the development of meningiomas

Currently, the etiology of meningiomas remains unknown; however, there are some established risk factors for their development such as exposure to ionizing radiation, genetic alterations and hormones.

Exposure to ionizing radiation is the strongest environmental risk factor identified so far, data on other risk factors such as cell phone usage and head trauma, remaining inconclusive [5, 8, 9]. Thus, several studies have demonstrated that repeated exposure to RT for other intracranial tumors or leukemia/lymphoma in infancy [5, 10, 11], and dental X-rays [12], are both associated with an increased risk for intracranial meningiomas. Similarly, low dose irradiation to treat *Tinea capitis* of the scalp was found responsible for single or multiple meningiomas with a life time risk of developing the disease of 2.3%, after a latency period of 35 years [13]. At higher doses, data exists for atomic bomb survivors who showed a greatly increased risk for meningioma [14]. In contrast, the role of head injury (e.g. trauma injury) in the development of meningioma is still a matter of debate [9]. Thus, results across different studies are not consistent, some reporting an increased risk of meningioma for both male and female among individuals who reported head trauma [15], whereas other studies did not confirm such association [16]. A potential mechanism leading to such association relates to the local alteration of the blood brain barrier, with a massive influx of cytokines into the extravasal space [5, 17].

Neurofibromatosis type 2 (NF2), an autosomal dominant disorder often associated with a cytogenetic deletion of the long arm of chromosome 22 at the q12 cytoband, is the most well-defined genetic condition associated with an increased risk for developing meningioma and schwannoma [5]. In this regard, most NF2 patients develop meningiomas which typically present earlier in life, with a high frequency of multiple lesions, particularly when compared to sporadic cases [8, 18]. The *NF2* gene is a tumor suppressor gene which codes for the merlin (schwannomin) protein, a molecule that builds a link between the cell membrane and the actin cytoskeleton (see section 2.1.1 for more detailed description of the merlin protein).

Finally, endogenous and exogenous hormones have also been proposed as a potential risk factor for meningioma [8, 9], because of the clear female predominance, as well as the association reported between meningioma and breast cancer [19], pregnancy, the menstrual cycle and menopause [20-22]; in addition, the expression of estrogen and progesterone receptors (PRs) on meningioma cells may further support the role of hormones in meningioma development [9]. In this regard, the changes observed in the size of meningiomas during

periods of a relative excess of progesterone, such as the luteal phase of the menstrual cycle and pregnancy, strongly support the progesterone dependency of these tumors [5]. Despite this, it should be noted that the specific role of sex hormones in meningioma tumorigenesis is still not fully clarified, and little evidence has been found so far which indicates that exogenous hormone exposure (e.g. usage of hormonal contraception or hormonal replacement therapy) might increase the risk for meningioma growth [21-23].

1.3. Localization, diagnosis and treatment of meningiomas

As the anatomical distribution of meningiomas is paralleled by the normal distribution of arachnoidal cells, meningiomas are found in several parts of the brain and the spinal cord [8]. Overall, the majority of meningiomas are intracranial tumors, with up to 60% being located in the convexity, parasagittal, tuberculum sellae, and sphenoid wing regions. Less common tumor localizations comprise the olfactory groove, the cavernous sinus, the falx, the lateral ventricle, the tentorium, the cerebellopontine angle, the middle fossa, and the orbita [5, 6, 24]. Spinal tumors are found in around 12% of patients with meningioma, and they are most commonly localized in the thoracic region [5, 6, 24].

As for other brain tumors at presentation, the clinical signs and symptoms associated with an underlying meningioma are directly related to the size and localization of the tumor. Despite this, general CNS-associated symptoms such as personality changes, neuropsychological deficits, headache, aphasia, sensory-motor or visual symptoms, as well as seizures, frequently occur [3, 24]. Computerized tomography (CT) and magnetic resonance imaging (MRI) are the most common minimally invasive diagnostic tools in cases where the presence of a meningioma is suspected. Meningioma images are usually isointense to the cerebral cortex and exhibit strong enhancement with contrast-based imaging [3]. Despite the fact that MRI has advantages over CT in assessing the characteristics of soft tissues, its combination with CT gives additional information about e.g. bone infiltration, allowing for optimal planning of subsequent surgery and radiation therapy [25]. Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) provide additional information about the cellular processes and biological characteristics of the tumor, which may be particularly useful in skull base meningiomas that are difficult to visualize by standard CT and MRI techniques [24, 26]; however, neither SPECT nor PET are currently used routinely for the diagnosis and follow-up of meningioma patients.

Once the diagnosis of meningioma has been established, treatment depends on tumor size and localization, the associated symptoms, age and the performance status of the patient [8]. At present, surgery remains the main treatment modality for meningioma patients [18]. Complete surgical resection of the tumor alone cures the majority of meningioma patients and complete tumor exeresis is associated with the longest progression-free and overall survival rates [27]. However, a complete resection of the tumor might not be achieved in some patients, especially in those cases with more difficult tumor localizations (e.g. meningiomas involving the cavernous sinus, the petroclival region, the posterior region of the superior sagittal sinus, or the optic nerve sheath) and spread to the surrounding tissues (e.g. the bone and/or the brain). In such cases, partial tumor removal followed by observation, adjuvant radiotherapy (RT), or in rare cases, systemic chemotherapy, are recommended [5]. Partial tumor resection is associated with poorer recurrence-free survival rates of around 63% at 5 years, 45% at 10 years, and 9% at 15 years [28]. In this regard, it should be noted that retrospective data based on a series of 140 patients demonstrated improved progression-free survival rates of around 89% at 5 years and 77% at 10 years in patients receiving adjuvant radiotherapy, after partial resection of benign meningiomas [29]. RT is also recommended for recurrent disease and cases showing a more aggressive tumor histopathology. In recent years, stereotactic radiosurgery (SRS) has become an alternative option for recurrent or partially resected meningiomas, and for patients in whom surgery is not an option because of the tumor's localization/infiltration profile [5, 8]. In one of the largest series of histologically benign meningiomas receiving gamma knife radiotherapy, high 5- and 10-year progression-free survival rates of 95.2% and 88.6%, respectively, have been reported [30].

Other alternative treatment modalities have also been investigated, particularly for non-resectable meningiomas, recurrent disease, and WHO grade II/III patients, in whom therapy complementary to surgery and RT are needed for curing the disease. In this regard, although several different chemotherapeutic agents have been evaluated, most have failed to show consistent efficacy, except for hydroxyurea [31]. In addition, several drugs targeting growth factor receptors (e.g. EGFR, PDGFR and VEGFR), hormone receptors (e.g. PRs) and their associated intracellular signaling pathways, have been investigated [32], and several phase II and phase III trials are currently underway in meningioma patients [8, 32]. Imatinib, sunitinib and tandutinib are some of the PDGFR inhibitors being investigated. However, treatment with imatinib, for example, demonstrated modest anti-tumoral activity when it was used alone [33] or in combination with hydroxyurea [34]. Bevacizumab, an anti-VEGF antibody demonstrated modest activity against meningiomas, being only able to induce partial remissions of the tumor [35, 36]. Of note, this and other VEGF and VEGFR inhibitors may also aid in reducing the

peritumoral edema, therefore also decreasing the morbidity associated with the tumor lesion [32]. Based on the high prevalence of elevated PRs expression in meningiomas, the PR antagonist mifepristone (RU486) has also been investigated with promising results in smaller studies, but a disappointing efficacy in a prospective multicenter study [32, 37]. In turn, interferon (IFN)-α [38, 39] and some somatostatin analogs (e.g. sandostatin) [40] are among those biologic agents showing a promising response profile in patients with recurrent meningiomas.

Based on all the above, future therapies for patients at risk of not being cured with conventional surgery and/or RT will most probably include combinations of targeted molecular agents, which will most likely be accomplished only through continued progress in the identification and understanding of genetic and biological changes associated with meningiomas [18, 32].

1.4. Histopathological classification of meningiomas

Although the majority of meningiomas are benign tumors, they display a surprisingly broad spectrum of clinical and histological features [6]. From the histopathological point of view, meningiomas are currently classified according to the WHO grading system, into three major (prognostic) categories: benign (WHO grade I), atypical (WHO grade II), and anaplastic (WHO grade III) meningiomas. Such histopathological grading system uses several different criteria which include the type of tumor cell, its mitotic activity, the cellularity of the tumor, and the presence of both necrosis and/or brain invasion (Table 1) [3, 4].

Table 1. World Health Organization (WHO) grading system for meningiomas. (Adapted from Riemenschneider et al. *Lancet Neurol* 2006 [3], Mawrin et al. *J Neurooncol* 2010 [4] and Saraf et al. *The Oncologist* 2011 [8])

WHO grade	*Frequency*	*Recurrence rates*	*Histopathological variants*	*Pathologic features/criteria*
Grade I	80-90%	7-20%	Meningothelial Fibrous (fibroblastic) Transitional (mixed) Psammomatous Angiomatous Microcystic Secretory Lymphoplasmacyte-rich Metaplastic	Pleomorphic Occasional mitotic figures Histological variants other than clear-cell, chordoid, papillary, or rhabdoid meningiomas Absence of criteria for atypical and anaplastic meningioma
Grade II	5-15%	30-40%	Atypical Clear-cell Chordoid	Any of three criteria: Mitotic index ≥4 mitoses/10 HPF ≥ 3/5 of the following variables: ° Increased cellularity ° Small cells with high N:C ratio ° Prominent nucleoli ° Uninterrupted patternless or sheet-like growth ° Foci of spontaneous necrosis Brain invasion
Grade III	1-3%	50-80%	Anaplastic (malignant) Rhabdoid Papillary	Either of two criteria: Mitotic index ≥20 mitoses/10 HPF Anaplasia

Abbreviations: HPF, high-power magnification fields; N:C ratio, nuclear:cytoplasmatic ratio.

1.4.1. WHO grade I/benign meningiomas

WHO grade I/benign meningiomas represent around 90% of all meningiomas and consist of multiple histopathological variants. From all histopathological subtypes, meningothelial, fibroblastic, and transitional meningiomas are the most commonly diagnosed [6]. By definition, these tumors do not have brain invasion, they do not fulfill criteria for atypical or anaplastic tumors [4], they have pleomorphic features and occasional mitotic figures [8, 24]. Although this WHO category is classically defined as being benign, it is associated with a high degree of intraclass variability, also reflected on different recurrence rates that can range from 7% to 20% [8], and that end up representing more than 50% of all meningioma recurrences.

1.4.2. WHO grade II meningiomas

WHO grade II tumors include 5-15% of all meningiomas. Typically, grade II meningiomas show a greater mitotic activity (≥4 mitoses per 10 high-power fields; HFP) than grade I tumors and they display at least three of the following five features which define a meningioma as being atypical: greater cellularity, small cells with a high nucleus-to-cytoplasm ratio, prominent nucleoli, uninterrupted patternless or sheet-like growth, and focus of necrosis [4, 6]. In the absence of the above cellular criteria for WHO grade II, presence of brain invasion also qualifies for this WHO subtype of meningiomas, since it is associated with both recurrence and mortality rates similar to those of atypical meningiomas [4, 24]. Finally, chordoid and clear cell meningiomas, which have a more aggressive clinical course, with a higher recurrence rate than histologically benign/grade I tumors, are also classified as grade II meningiomas. Five-year recurrence rates for grade II meningiomas are of 30-40% for those patients undergoing complete tumor resection. Among these tumors, the presence of a high mitotic activity and micronecrosis with pseudopalisading are strong risk factors associated with a higher risk of recurrence [3, 8].

1.4.3. WHO grade III meningiomas

Anaplastic and other malignant histopathological subtypes of meningiomas are classified as WHO grade III tumors, accounting for a small fraction (1-3%) of all cases. Grade III tumors show a mitotic index of ≥20 mitoses/10 HPF or the presence of clear anaplastic features, defined as being similar to those of a carcinoma-, melanoma- or sarcoma-like histology [6]; in addition, outer areas of spontaneous necrosis are also commonly observed in this tumor subgroup. Because of the clinically aggressive nature of the rhabdoid and papillary histopathological variants of meningiomas, they are both also designated as WHO grade III tumors. Overall, grade III tumors show clinical characteristics which are similar to those of other malignant neoplasms, including higher frequencies of local invasion, recurrence, and metastasis; prognosis of grade III meningiomas is poor, with recurrence rates of 50-80% after complete surgical resection and a median survival of less than 2 years [3, 6, 8].

1.5. Prognostic factors and outcome of meningiomas

Despite the usually benign nature of most meningiomas and the good long-term prognosis associated with these tumors, recurrences do occur in a significant proportion (10-25%) of cases, representing the most relevant clinical complication of this group of brain

tumors [3, 6]. Of note, multiple studies also indicate that survival times are shorter in patients with meningioma than in age- and sex-matched controls, particularly for those patients showing tumor recurrence [8, 41]. Therefore, identification, already at diagnosis and diagnostic surgery, of those patients which present a high-risk of recurrence *vs.* those who are cured, represents a major challenge. Thereby, many studies have investigated clinical and molecular/genetic features of meningiomas that might contribute to better understand the behavior of the disease and predict patient outcome, for more accurate prognostic stratification of patients already at diagnosis, into distinct subgroups with different risk of tumor recurrence [24]. If such patients with a high-risk of recurrence could be identified earlier, a closer follow-up and/or an alternative/complementary treatment strategy could be used for these cases [5].

Classically, the strongest prognostic factors for patient survival include the histopathological WHO tumor grade [6, 24] and the extent of the surgical resection of the tumor as evaluated by the Simpson grading system [8, 24, 27, 32, 41]. Accordingly, atypical/grade II and malignant/grade III meningiomas show higher recurrence rates and shorter survival times than benign/grade I tumors, with 5-year recurrence rates of approximately 40% and 80% *vs.* 7%, respectively, and a median recurrence-free survival of 11.5 and 2.7 years *vs.* >10 years, respectively [8, 24, 42]; despite this, it should be noted that in absolute numbers most recurrences still occur among benign/grade I meningiomas. In turn, following complete tumor resection, meningiomas have a recurrence rate of 10-30% at 10 years, whereas tumors with subtotal resection have a higher 10-year recurrence rate of 45-61% [28, 32]. Therefore, achievement of complete tumor resection remains a major goal of meningioma treatment which, among other factors, depends on tumor localization [8]. In line with this, tumors of the convexity can be usually cured by surgical resection alone, whereas skull-based tumors, especially those localized in the petroclival region or with involvement of the cavernous sinus or the orbit, often have a more unfavorable outcome [8, 41, 43]. Other tumor-associated factors that have an adverse impact on patient outcome, as well as on the efficacy of surgery for curing meningioma, include a large tumor size and the presence of brain or bone invasion, both features being associated with higher recurrence rates [5, 24].

Additional patient features which have been associated with a poorer outcome of meningiomas include younger age at diagnosis and male gender [8, 41, 44], although their independent prognostic value from other variables remains controversial. In this regard, it should be noted that while younger patients usually show more aggressive tumors and higher recurrence rates [41, 44, 45], older cases are associated with lower overall survival rates if not matched to age-associated expectancy life [5, 8].

In addition to all the above features, several other biologic markers have been identified as being associated with more aggressive disease and greater risk of recurrence in meningiomas. Among others, these include proliferation markers (MIB-1/Ki-67 and the percentage of S-phase tumor cells) [32, 42, 46-48], the telomerase activity and *hTERT* mRNA levels [49-53], which are associated with both a more advanced WHO grade of the tumor and a greater risk of disease recurrence. In addition, the pattern of expression of sex hormone receptors has also been recurrently associated with the prognosis of the disease; thus, whereas meningiomas expressing PRs show a low frequency of recurrence and a better overall prognosis, meningiomas with estrogen receptors or absence of PRs, are usually associated with higher recurrence rates [5, 24]. More recently, immunohistochemistry (IHC) staining for the osteopontin protein, an integrin-binding protein involved in proliferation, adhesion, migration, and angiogenesis, has also been reported as a valuable marker to predict the risk for 'early' recurrence within WHO grade I meningiomas [54]. Similarly, VEGF has also been associated with adverse disease features in meningiomas patients such as a higher frequency of brain edema, anaplastic and atypical meningiomas, and a higher risk of recurrence [55-57]. In this regard, Barresi et al. [58] further suggested that the ratio between the pro-angiogenic VEGF and the anti-angiogenic Semaphorin3A (SEMA3A) markers may better reflect the status of regulation of neo-angiogenesis in meningiomas, a high VEGF/SEMA3A ratio being strongly associated with disease recurrence and high histological grades, as well as greater tumor proliferation indices and microvessel density in the tumor.

Despite all the above prognostic factors, chromosomal and genetic/molecular alterations have been a major area of research as regards the identification of markers with independent prognostic value. Thereby, several chromosomal alterations have been associated with higher recurrence rates and shorter survival. Accordingly, deletions of chromosomes 1p [59-62] and/or 14q/monosomy 14 [60, 63, 64] have been identified as strong independent prognostic factors in meningiomas, as it will be discussed in more detail in the following subsection of this introduction. Other chromosomal alterations which have also been related with higher risk of recurrence include losses of chromosomes 10 [65] and 9p [66, 67], and gains of chromosomes 22 [68] and 1q [69, 70]. In addition, while some specific gene-expression profiles have been identified to be associated with tumor aggressiveness and recurrence [71-75], the independent prognostic impact of such profiles still deserves to be validated in other prospective studies in large series of meningioma patients.

Despite all the above, and the fact that a few prognostic scoring systems have been proposed in the latest decade, at present there is still no widely-accepted prognostic classification of meningioma that is able to predict at diagnostic surgery which patients are

cured and who are at a high-risk of recurrence and would therefore benefit from a more closer follow-up and/or complementary/alternative treatment measures.

2. THE GENETICS OF MENINGIOMAS

In contrast to modern WHO classification of e.g. hematological malignancies, the current WHO classification of meningiomas is still mostly based on morphological and histopathological criteria. Since meningiomas display a wide spectrum of histopathological patterns, associated with an equally wide range of biological features and an heterogeneous clinical behavior, even within the same WHO grade, additional criteria and biomarkers are needed to further enhance the prognostic value of the current WHO grading and improve the management of individual meningioma patients. In this regard, it should be noted that the development of a combined histopathological and molecular classification of meningiomas emerges as a new attractive approach [76, 77].

In recent years, research on meningiomas has largely focused on the understanding of the molecular mechanisms that underlie tumorigenesis and disease progression. As a consequence of such studies, substantial insight into the molecular biology of these tumors has been achieved and several chromosomal regions and candidate genes have been identified. Monosomy 22/del(22q) is by far the most frequent cytogenetic event in meningiomas, potentially occurring at early stages of the disease. Apart from the genetic alterations on chromosome 22, other isolated chromosomal alterations, together with more complex karyotypes, have been reported at relatively high frequencies in meningiomas, usually in association with a more aggressive tumor behavior [32]. Although, malignant progression from low- to high-grade meningiomas may occur, it is still considered controversial [4]. This is due to the fact that data which is used to explain stepwise progression (cumulative acquisition of chromosomal gains and losses, leading to more aggressive subclones) typically derives from the cytogenetic analysis of different tumors of distinct grades and from different patients [62, 78]. In contrast, studies addressing this question through the follow-up of patients showing tumor recurrence reported mostly unchanged grade at recurrence [79]; despite this, it is well known that a few meningiomas can become progressively more aggressive despite their original benign status, and some compelling evidence of malignant progression has also been reported in the literature [80]. In this regard, meningiomas that present with complex genetic alterations, and that include both atypical and anaplastic meningiomas, as well as some grade

I/benign tumors, could potentially correspond, at least in part, to more advanced tumors than those showing isolated monosomy 22/del(22q) or other isolated chromosomal changes. In this section we will review the most relevant genetic and molecular alterations that have been described so far in meningiomas, specially emphasizing those chromosomes, genes and signaling pathways most frequently altered in these tumors.

2.1. Genetic alterations of chromosome 22 in meningiomas

2.1.1. The *NF2* gene and the merlin protein

A high proportion of all meningiomas have long been shown to contain recurrent genetic alterations involving chromosome 22 and the *NF2* tumor suppressor gene coded in the long arm of chromosome 22 (22q) [81-83]. Accordingly, monosomy 22 is the most frequent genetic abnormality in meningiomas and it is found in around half of the cases. In turn, the great majority of NF2-associated meningiomas, and between 40-70% of sporadic meningiomas, display allelic losses (loss of heterozygosity, LOH) at the 22q12.2 chromosomal region, where the *NF2* gene is encoded [4, 77]. Additionally, up to 60% of these meningiomas carry inactivating mutations in the remaining *NF2* allele [4, 84, 85], consistent with the classical two-hit hypothesis of tumor suppressor gene inactivation [83] originally proposed by Knudson [86], in which the two alleles of a particular tumor suppressor gene must be rendered ineffective for the cell to escape normal regulatory control checkpoints. Most *NF2* mutations consist of small insertions, deletions, or nonsense mutations affecting the splicing sites [4, 87]. Despite Lomas et al. [88] have reported that the NF2 gene may be alternatively inactivated in meningiomas by aberrant promoter methylation, an analysis of 40 CpG sites within the 750 bp surrounding the promoter regions of the *NF2* gene, showed methylation at only one CpG site in 1 out of 12 tumor samples [89] and, in another study, the *NF2* gene itself was methylated in only 1 out of 21 tumors [90]. Therefore, as regards epigenetic silencing, studies on the *NF2* gene indicate that methylation of the *NF2* promoter does not play a major role in meningioma development [89-91].

As the frequency of *NF2* mutation is roughly equal among the different WHO grades, it has been considered a relevant genetic alteration in tumor initiation rather than in malignant progression, representing an early event in meningioma tumorigenesis [3, 4, 77]. Biallelic inactivation of the *NF2* gene results in loss of the merlin protein (also known as schwannomin), the product of the *NF2* gene [76]. Amino acid analysis has revealed that merlin is part of the 4.1 family of proteins (i.e. the ERM family: ezrin, radixin and moesin), which link integral

membrane proteins to the cytoskeleton, being involved in the regulation of cell growth, proliferation and motility [92]. Meningioma-associated *NF2* mutations commonly result in a truncated, non-functional protein, which may lead to abnormal cell growth and motility through destabilization of adherens junctions [93]. The main characteristic of cells lacking the NF2 protein product is the loss of contact-mediated inhibition of cell proliferation [85]. Additionally, loss of merlin activity has been associated with elevated levels of ErbB receptors in primary Schwann cells, which control the activity of the downstream mitogenic signaling pathways (e.g. Ras/Raf/MEK/ERK and PI3K/AKT); altogether, these findings support the role of merlin in tumorigenesis [94]. In line with this hypothesis, mice which are heterozygous for *NF2* mutations more frequently develop metastatic tumors [95], and both *in vivo* and *in vitro* re-expression of wild type merlin leads to reduced tumor growth and decreased cell motility [77, 92].

From a structural point of view, merlin exerts its growth-regulatory functions through its cell surface glycoprotein-binding domain (FERM domain), which mediates interactions with several cell surface signaling proteins (e.g. β1 integrin and CD44) and cytoskeleton-regulating proteins (e.g. paxillin, actin and syntenin) [85, 92, 96-99]. Among these merlin-binding partners, the interaction with the CD44 glycoprotein is one of the most intriguing, as merlin and CD44 antagonize each other's function [85, 92, 99]: merlin mediates contact-induced inhibition of cell growth through its interaction with the cytoplasmic tail of CD44. Morrison et al. [97] proposed that merlin and CD44 form a molecular switch between growth-promoting and growth-inhibiting conformations, where external signals for growth inhibition (e.g. increased cell density) lead to consequent merlin activation (dephosphorylation), indicating that merlin exerts its tumor suppressor function, at least in part, by negatively regulating the function of CD44 [100].

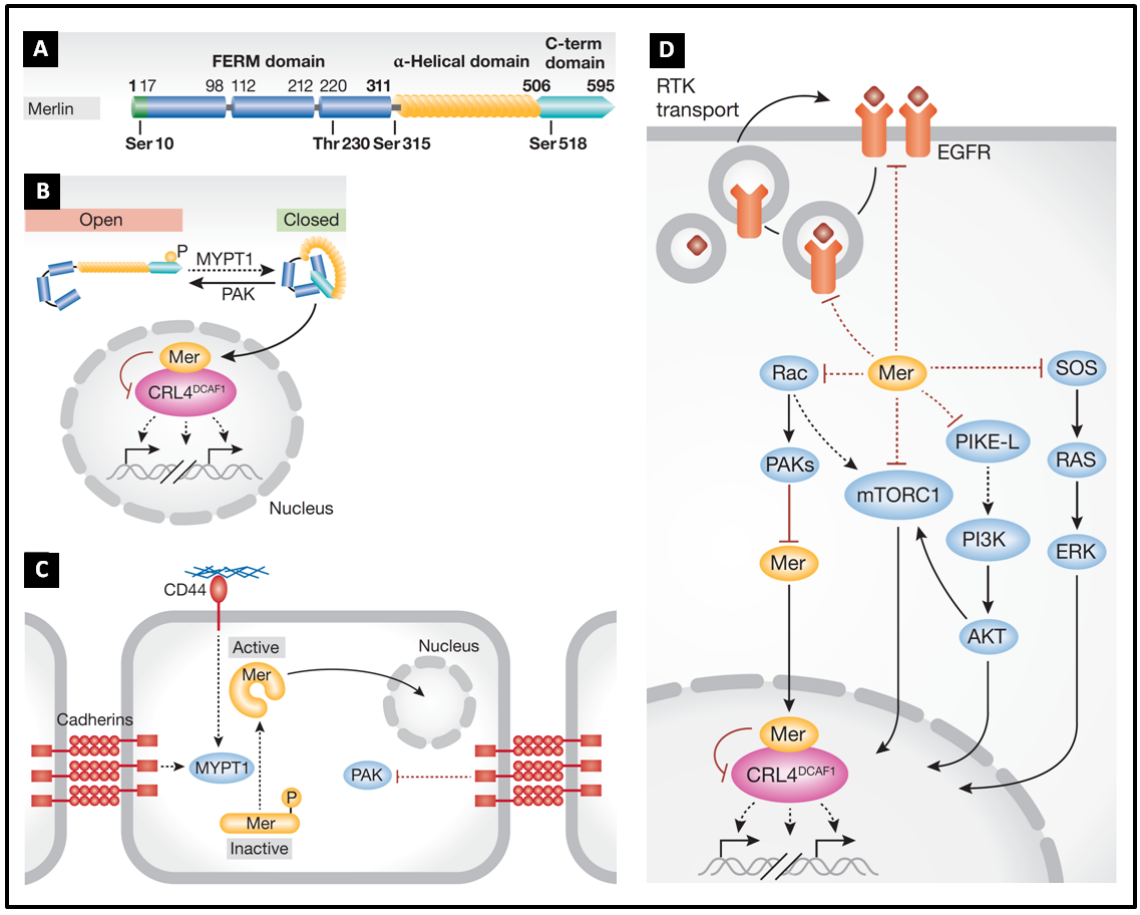

Figure 1. Schematic representation of the structural organization of the merlin protein domain, its activation by phosphoregulation and its downstream signaling pathways. The convergence of several upstream adhesion receptors regulates Merlin (Mer) activation and subsequently controls downstream mitogenic pathways. (**A**) Merlin has a domain organization consisting of an amino-terminal (N-term) FERM domain, an α-helical coiled-coil domain and a carboxy-terminal (C-term) hydrophilic tail; merlin phosphorylation sites are indicated in the figure. (**B**) The merlin's dephosphorylated and closed form is active, promoting nuclear translocation and tumor suppression through growth inhibition. Phosphorylation at Ser 518 renders the protein inactive in its putatively open form. (**C**) The assembly of cell-to-cell adhesions and CD44 activation by hyaluronic-acid-rich matrix activates MYPT1, which dephosphorylates Merlin Ser 518 and maintains it in a closed and active conformation. (**D**) Merlin can affect a variety of mitogenic signaling pathways, including Rac-PAK signaling, mTORC1, EGFR-Ras-ERK and the PI3K-Akt pathway. **Abbreviations:** MYPT1, myosin phosphatase targeting subunit 1; PAK, p21-activated kinase; PKA, protein kinase A; Akt, protein kinase B; CRL4, cullin-ring E3 ligase 4; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; mTORC1, mammalian target of Rapamycin complex 1; PI3K, phosphatidylinositol 3-kinase; Rac, Ras-related C3 botulinum toxin substrate; Ras, rat sarcoma. (Modified from Li W. et al. EMBO reports 2012 [101])

Since merlin functions include linking membrane proteins to the cytoskeleton, it has been hypothesized that alterations in merlin may substantially affect cell shape and might favor the appearance of a more mesenchymal-like phenotype rather than the normal epithelioid one, which is more commonly observed in wild type *NF2* meningiomas [4, 76]. Of note, several studies have reported different frequencies of *NF2* mutation in meningiomas displaying distinct histopathological features; in this regard, abnormalities of chromosome 22q

are more frequently observed in transitional and fibrous meningiomas than in the meningothelial variant [89, 102-105]. These findings further support the notion that *NF2* mutation and/or deletion could play a preferential role in meningiomas with a mesenchymal-like phenotype. In addition, an association between the *NF2* gene and tumor localization has also been reported. Thus, Kros et al. [103] demonstrated that tumors of the convexity are more prone to have *NF2* alterations than anterior cranial-based tumors, and Clark et al. [106] recently correlated meningiomas with mutant NF2 and/or chromosome 22 loss with tumor localization in the cerebral and cerebellar hemispheres. In the latter study, authors reported distinct genome profiles for meningiomas depending on the presence *vs.* absence of *NF2* mutation, non-*NF2* mutated tumors frequently showing mutation of other genes (e.g. *TRAF7*, *KLF4*, *AKT1* and *SMO*) [106]. In line with the unique features associated with NF2 mutation in meningiomas we have also recently observed these occur mostly in postmenopausal women [87].

2.1.2. Other candidate genes coded in chromosome 22

Although *NF2* is the most frequently altered gene in chromosome 22, the frequency of deletions at this chromosomal region exceeds by far that of *NF2* mutations in meningioma, suggesting that other genes encoded at chromosome 22 may also be involved in meningioma tumorigenesis. In this regard, an early report found *BAM22* - a gene from the β-adaptin family coded at chromosome 22q12 - to be inactivated in 9 out of 71 meningiomas [107], and another more recent report found reduced expression of the *BCR* (breakpoint cluster region) gene coded at chromosome 22q11 in meningiomas with 22q LOH [108], further supporting the existence of candidate genes other than *NF2* in the pathogenesis of meningiomas.

Tissue inhibitors of metalloproteinases (TIMP) are proteins that regulate matrix metalloproteinases (MMP) and thereby, also help to regulate cell proliferation, apoptosis, and angiogenesis [76]. The *TIMP3* gene, coded at chromosome 22q12, is currently the best understood tumor suppressor gene as pertaining to epigenetic regulation of meningiomas [91], and was recently associated with a more aggressive and higher-grade meningioma phenotype [91, 109, 110]. Barski et al. [109] reported inactivation of *TIMP3* via promoter hypermethylation to be present in 67% of anaplastic meningiomas *vs.* 22% and 17% of atypical and benign meningiomas, respectively; similarly, Bello et al. [110] found *TIMP3* inactivation in 40% *vs.* 18% of grade II-III *vs.* grade I meningiomas. In line with these findings, TIMP1 has also been implicated in the aggressive behavior and invasion of meningiomas [111]. As TIMP1 inhibits the activity of MMP9, a few studies have investigated the TIMP1/MMP9 balance in meningiomas [112-116]: reduced secretion of TIMP1 has been found to be associated with an

increase in the infiltrative capacity of meningiomas [113], while high expression of MMP9 has been associated with higher histological grades, proliferation index and risk of recurrence, supporting a potential prognostic value for these two markers [114-116].

2.2. Other relevant chromosomes in meningiomas

In addition to monosomy 22/del(22q), other isolated and combined chromosomal alterations have also been identified in meningiomas. Thus, losses of chromosomes 1p, 10, 14/14q, and less frequently also of chromosomes 6q, 9p, 18q and the sex chromosomes, together with gains of chromosomes 1q, 9q, 12q, 15q, 17q, and 20q, have all been recurrently reported in a variable proportion of cases that overall account for around 30% of all meningiomas [4, 32, 62, 75, 78, 80, 117, 118]. Despite several candidate genes have been proposed to be targeted by these chromosomal alterations still, the specific relevant genes involved, remain most frequently unknown, as discussed below per chromosome.

Genetic alterations of chromosome 1. Chromosome 1p deletions comprise the second most common chromosomal abnormality in meningiomas [84, 119]. Del(1p) is typically associated with more aggressive meningiomas and disease recurrence [84, 119]. In fact, the frequency of del(1p) has been reported to be higher in grade II/III tumors, being found in 13-26% , 40-76%, and 70-100% of grade I, grade II and grade III meningiomas, respectively [77, 120]. From the clinical point of view, loss of 1p is also associated with higher tumor recurrence rates [59, 60]. The most frequently targeted regions involve the chromosomal 1p33-34 and 1p36 cytobands [118, 119, 121], where several candidate (target) genes have been identified. Among other genes, these include the *TP73, CDKN2C*, *EPB41*, *GADD45A*, and *ALPL* genes [77]. However, current results do not support a key role in tumorigenesis and/or malignant progression for most of these genes as meningioma-specific tumor suppressors as they all failed in showing consistent structural alterations. As an example, only one mutation has been found in the *CDKN2C* gene (a cell cycle control gene encoding $p18^{INK4C}$ at 1p32), at the same time no hypermethylation-mediated inactivation of this gene has been found [122, 123], demonstrating that p18 is unlikely to play a relevant role in the pathogenesis of meningiomas. Similarly, LOH and expression analysis failed to find losses of *EPB41* (a gene encoding for the 4.1R protein at 1p33-p32) and *GADD45A* (at 1p31.2) [124]. In contrast, although mutation of the *TP73* gene (a structurally and functionally *TP53* homologous gene located at 1p36.33) has not been found, methylation-mediated inactivation of *TP73* has been recurrently reported as

frequent in meningiomas [110, 125, 126]. Similarly, evidences exist about the potential role of the *ALPL* gene encoding for an alkaline phosphatase enzyme at 1p36.1-34, as a tumor suppressor gene, because loss of chromosome 1p in meningiomas is strongly associated with loss of alkaline phosphatase activity [78, 127], such alteration being reported to be a predictor of meningioma recurrence [128]. Despite this, mutational analysis of *ALPL* has not been reported in the literature.

On the other hand, gains of chromosome 1q have been reported in around 60% of atypical meningiomas, such gains mostly targeting two chromosomal regions: 1q25.1 and 1q25.3 to 1q32.1 [69, 70]. From the prognostic point of view, gains of chromosome 1q have been recurrently associated with shorter progression-free survival [69, 70]. However, detailed examination and identification of those genes with oncogenic potential, which may be coded in this chromosome arm, still deserves further investigation.

Genetic alterations of chromosome 6. Genetic losses involving the long arm of chromosome 6 are a relatively common finding in meningiomas, particularly among high grade tumors, reported frequencies ranging from 9% of grade I to 25-33% of grade II and 50-63% of grade III meningiomas [75, 118, 129]. A common deleted segment at chromosome 6q includes the 6q24.1-qter region, where the *ESR1*, *IGF2R* [118], *DLL1*, and *CTGF* [71] cancer-associated genes are encoded; however, the functional role of these genes and the impact of their alterations in meningiomas are still far from being fully understood. In addition to del(6q), Pérez-Magán et al. [71] also reported overexpression of the histone cluster 1 genes coded at chromosome 6p (e.g. the *HIST1H1c* gene) in 27% and 89% of primary and recurrent meningiomas, respectively. Based on their results, these authors suggested that physical interaction of the H1.2 protein could be involved in epigenetic regulation of gene expression by maintaining specific DNA methylation patterns, hypothesizing that it may suppress p53-dependent, p300-mediated chromatin transcription by blocking chromatin acetylation.

Genetic alterations of chromosome 9. Genetic alterations of chromosome 9 are a relatively frequent finding in meningiomas, particularly in malignant tumors [67, 84]. In more detail, losses at chromosome 9p have been reported in 5-17% of grade I, 18-52% of grade II, and 38-74% of grade III meningiomas [67, 77, 122]. While the target genes and the precise tumorigenic mechanisms of other chromosomal alterations in meningiomas are still unclear, the role of chromosome 9 in the development of malignant meningiomas is better defined as it has been associated with three tumor suppressor genes coded at chromosome 9p21: *CDKN2A/p16*$^{INK\alpha}$, *p14*^{ARF} and *CDKN2B/p15*^{ARF}. Proteins coded by these three genes are all well

known proteins which play an important role in cell cycle regulation and the apoptosis pathways; $p16^{INKa}$ and $p15^{ARF}$ regulate cell cycle progression at the G1/S-phase checkpoint by inhibiting cyclin-cdk complexes, whereas p14ARF regulates apoptosis through blocking Mdm2-mediated degradation of p53 [76, 77] (see section 2.5.1. for more detailed information on the pRb/p53 pathways in meningiomas). In addition, both homozygous deletions and somatic mutations of these genes have been reported in anaplastic meningiomas, supporting the notion that inactivation of cell cycle regulation is an important feature in malignant progression of meningiomas. As an example, Boström et al. [122] found homozygous deletions of *CDKN2A*, *CDKN2B*, and *p14ARF* in 46% of anaplastic *vs.* 3% of atypical meningiomas. In a similar way, Goutagny et al. [130] have recently shown by SNP-arrays that the most frequent genomic alteration of meningiomas upon progression to grade III was loss of *CDKN2A*/*CDKN2B*. Additionally, inactivation through hypermethylation of CpG islands has also been shown to occur in a smaller proportion of meningiomas, including hypermethylation of *CDKN2A* in 8-17% of cases, $p14^{ARF}$ in 4-13%, and *CDKN2B* in 4% of these tumors [110, 131, 132]. Of note, Amatya et al. [133] found hypermethylation of the $p14^{ARF}$ gene to be associated with tumor grade as it was present in 9% of benign, 20% of atypical and 50% of anaplastic meningiomas. All such correlations also appear to translate into a prognostic impact, since meningiomas with 9p21 losses have a considerably shorter survival and worst outcome than other cases showing no alterations at chromosome 9p21 [67].

Genetic alterations of chromosome 10. Losses of part or the whole chromosome 10 are present in a significant proportion of meningiomas, their frequency increasing from grade I (5-12%) to grade II (29-40%), and grade III (40-58%) tumors [77, 129, 134], some studies reporting even higher frequencies [65, 135]. In addition, LOH at specific regions of chromosome 10 have been associated with both a poorer survival and higher recurrence rates [65]. Some of the potential candidate genes encoded in such chromosomal regions (e.g. 10q23-q25) include the *PTEN*, *MXI1*, and *DMBT1* genes. However, it should be noted that despite being extensively investigated, several studies have failed to identify frequent/recurrent mutations of these genes in meningiomas, e.g. mutation of *PTEN* at 10q23.3 [135-137].

In addition, hypermethylation of the promoter of the *MGMT* (O6-methylguanine–DNA methyltransferase) gene - a gene involved in the sensitivity to temozolomide (TMZ) therapy in gliomas - at chromosome 10q26.3, has been reported in ≤11% of meningiomas [131, 132, 138, 139], providing no biological rational that would support the use of TMZ in the treatment of these tumors.

More recently, Dobbins et al. [140] identified a new susceptibility locus for meningioma at 10p12.31, which encompasses the *MLLT10* gene (encoding myeloid/lymphoid or mixed-lineage leukemia translocated to 10); this gene is involved in chromatin remodeling and modulation of transcription. However, further investigations about the potential role of this gene in meningiomas are still needed.

Genetic alterations of chromosome 14. In a similar way to chromosome 1p losses, partial deletion and complete losses of chromosome 14 are common events in meningioma, being typically found in a association with more aggressive tumors that also carry chromosome 1 losses [4]. Overall, monosomy 14/14q^{-} represents the third most common chromosomal alteration in meningiomas, being found in up to 31% of grade I, 40–70% grade II, and up to 100% of grade III meningiomas [63, 64, 129, 141, 142]. From the prognostic point of view, chromosome 14q status has been identified as a prognostic indicator for tumor recurrence [45, 63, 64]. Because of this, among other chromosome 14 regions, the 14q32 region has been suggested to be potentially relevant for meningioma progression; in this regard, Zang et al. [143] have identified the *maternally expressed gene 3 (MEG3)* gene in this chromosomal region, as a candidate tumor suppressor gene with anti-proliferative activity. *MEG3* is an imprinting gene that encodes for a non-coding RNA. In meningiomas, loss of *MEG3* expression and its deletion at the genomic DNA, as well as the degree of methylation of its promoter, have all been associated with higher tumor growth. In turn, functional studies showed that MEG3 mediates its anti-tumoral effect through inhibition of DNA synthesis, colony formation and proliferation of meningioma cell lines. In addition, MEG3 has also been found to transactivate p53, another tumor suppressor gene involved in cell signaling pathways often dysregulated in anaplastic meningiomas [143]. Altogether, these findings suggest that MEG3 may have a significant role as a novel long non-coding RNA tumor suppressor in meningiomas. Recently, the *AKT1* gene (coded at 14q32) has been reported to be mutated in meningiomas lacking *NF2* mutations [106, 144], the reported mutation (E17K) resulting in constitutive AKT1 activation [144]; therefore, the AKT1 gene has become one of the most attractive target genes for cases with monosomy 14/del(14q).

In another study, Lusis et al. [145] also identified the *N-Myc downstream-regulated gene 2 (NDRG2)* as a gene commonly inactivated during meningioma progression to be a potential tumor suppressor gene coded at chromosome 14q11.2. Thus, *NDRG2* was found to be frequently downregulated at both the transcript and the protein levels in anaplastic meningiomas, and in a subset of lower-grade and atypical cases with an aggressive clinical behavior [145], as well as in recurrent meningiomas [146]. Reduced expression of *NDRG2*

appears to be closely associated with hypermethylation of its promoter [145]. Despite the precise mechanism of action of *NDRG2* remains largely unknown, this gene has been involved in the regulation of cell growth, differentiation and apoptosis [91].

Genetic alterations of chromosome 17. Chromosome 17 gains and/or amplification of the 17q21-qter chromosomal region have been recurrently reported, mostly in malignant meningiomas [117, 118, 129, 147, 148]. *RPS6K* (ribosomal protein S6 kinase; $p70^{S6K}$) is a proto-oncogene coded at chromosome 17q23 which has been reported to be overexpressed at the protein level [149]; however, amplification of this gene appears to occur only in a small subset of higher grade meningiomas, even when amplification of the loci adjacent to this gene is present [147]; these results suggest that other genes coded in the vicinity of *RPS6K* may be the main target for 17q amplification. In this regard, recent studies have also investigated the potential role of *STAT3* (coded at 17q21.2), showing a higher frequency of enhanced expression of *STAT3* with increasing tumor grade [150, 151]. Zhang et al. [150] further reported that constitutively active STAT3 was significantly associated with expression of vascular endothelial growth factor (VEGF), a major inducer of tumor angiogenesis, and Johnson et al. [152] suggested that the CSF itself may act as a stimulus for STAT3 phosphorylation/activation.

Genetic alterations of chromosome 18. Losses at chromosome 18q have been recurrently reported in meningiomas [73, 77, 118]; however, the specific target genes still remain to be identified. In this regard, Büschges et al. [153] investigated the *MADH2*, *MADH4*, *APM-1* and *DCC* tumor suppressor genes coded at chromosome 18q21, reporting only one missense mutation in *APM-1*; these results suggest that these genes might not be the target genes to be inactivated in 18q losses in meningioma. In contrast, the expression of the bcl-2 oncoprotein coded at 18q21.3, has been associated with both a higher tumor grade and recurrence rate [154-156].

In parallel, due to the role of the merlin protein in meningioma tumorigenesis, several studies have further investigated other members of the 4.1 family of membrane-associated proteins. These included the *DAL-1* (differentially expressed in adenocarcinoma of the lung) gene which encodes for the 4.1B protein and that has been claimed also to act as a potential tumor suppressor gene in meningiomas [157]. Thus, loss of heterozygosity of *DAL-1* at chromosome 18p11.32 was initially reported to occur in 60-76% of sporadic meningiomas [158, 159], independently of the histological grade, suggesting it could represent an early event in the pathogenesis meningioma. Interestingly, despite the precise mechanisms involved are still

unclear, Gerber et al. [157] provided the first molecular insights into the potential tumor suppressor role of the 4.1B/DAL-1 protein by showing it is involved in c-Jun-NH2-kinase (JNK)-mediated activation of the Src, Rac1, and MLK3 signaling cascades. JNK activation decreases cell growth through reduced expression of cyclin A, hyperphosphorylation of the retinoblastoma protein (Rb), and G0-G1 cell cycle growth arrest. However, more recent studies brought conflicting results about the potential role of the DAL-1/4.1B tumor suppressor gene. Thus, Yi et al. [160] reported that transgenic mice lacking *DAL-1* do not develop tumors and Nunes et al. [161] reported that only 12 (19%) out of 62 meningiomas had LOH of *DAL-1*, 11 of such 12 cases also showing LOH of the *NF2* gene. Altogether, these results suggest that *DAL-1* may be involved in progression rather than initiation of meningiomas. In turn, these authors [161] also found monosomy 18 and/or del(18p) to be present in 3/4 WHO grade II tumors *vs.* 2/13 WHO grade I meningiomas. Other recent reports observed no losses in the genomic regions containing the *DAL-1* gene [90, 144]. In another study based on the analysis of 83 meningiomas, a very low mutation frequency of the *DAL-1* gene was found, suggesting that epigenetic changes rather than genetic mutation, may be responsible for 4.1B/DAL-1 silencing in meningiomas [162].

2.3. The DNA methylation profile of meningiomas

Around 30% to 40% of all meningiomas do not show any significant genetic alteration, suggesting that other mechanisms could be involved in tumor development, at least in this subgroup of patients. In line with this hypothesis, epigenetic changes have been identified for at least one (methylated) gene in 77% of cases [91], supporting the notion that epigenetic alterations, including increased CpG island hypermethylation, may play an important role in meningioma tumorigenesis, particularly in association with malignant progression. As described above, hypermethylation involving the promoter regions of the *TIMP*-3 [109, 110], *TP73* [110, 125, 126], *MEG3* [143], *NDRG2* [145], *CDKN2A*, *p14^{ARF}*, and *CDKN2B* [110, 131-133] genes has been reported at variable frequencies in meningiomas. Furthermore, hypermethylation of the *HOXA (homeobox A)* genes at the chromosome 7p15.2 has also been reported in association with downregulation of tumor suppressor targets. In this regard, high-throughput genome-wide DNA methylation studies by Kishida et al. [163] and Di Vinci et al. [164] identified several genes to be potentially implicated in progression of meningiomas, including the *HOXA6* and *HOXA9* genes. Similarly, hypermethylation of *GSTP1* has also been reported in meningiomas [131, 132], in association with tumor grade: from 0% in WHO grade I

tumors to 32% and 54% in atypical and anaplastic variants [131]. Of note, the *GSTP1* gene encodes for a member of the glutathione-S-transferase family of proteins that play an important role in protecting cells from carcinogenic agents, by preventing DNA damage.

Interestingly, epigenetic alterations of genes involved in specific cell signaling pathways related with the pathogenesis of meningiomas, such as CpG island hypermethylation of the *RB1* (encoding the pRb tumor suppressor) [90, 131] and the *IGF2BP1* (encoding for Insulin-like growth factor 2 mRNA-binding protein 1, regulator of IGF2 translation) [163, 165] genes, has also been reported. Similarly, 15-30% of meningiomas show hypermethylation of *THBS1* (thrombospondin 1) [110, 131], a gene that inhibits angiogenesis by disrupting the motility of endothelial cells and inducing their death by apoptosis. In line with this hypothesis, Bello et al. [110] further found hypermethylation of the *THBS1* gene in 54% of grade III meningiomas, suggesting that inactivation of this gene could lead to neovascularization of atypical meningiomas and consequently, contribute to tumor progression. Another interesting gene found to be regulated by hypermethylation in 83% and 71% of grade II and III meningiomas, respectively, is the *WNK2* gene, a negative regulator of EGF-induced activation of the ERK/MAPK-pathway and downstream cell cycle progression [166].

2.4. Telomerase activity in meningiomas

Telomeres comprise repeated DNA sequences at the ends of chromosomes and they function to prevent chromosomal deterioration. Telomerase is defined as the reverse transcriptase activity that stabilizes chromosomal length by rebuilding the lost telomere repeat sequences; telomerase activity has been found to be often reactivated during tumorigenesis [84]. Activation of telomerase seems to play an important role in progression of meningiomas since both atypical and anaplastic tumors display increased telomerase activity and/or elongated telomeres once compared to benign WHO grade I tumors. Accordingly, telomerase activity has been reported in 3-21%, 58-92% and 100% of benign, atypical, and anaplastic meningiomas, respectively [49-51, 77], and it has been further associated with both a poorer outcome and a higher rate of tumor recurrence [49, 51, 52]. The main components of the telomerase complex are a reverse transcriptase (hTERT) and an integral RNA component (hTR). Expression of *hTERT* mRNA, rather than *hTR*, is best correlated with telomerase activity in meningiomas. In fact, it has been reported that hTERT expression might be a more sensitive marker than the telomerase activity *per se*, and Maes et al. [53] suggested that hTERT expression might be an early event in carcinogenesis, whereas the switch on the telomerase activity would emerge later.

Table 2. Most relevant genes in the pathogenesis of meningiomas and their chromosomal localization, type of genetic alteration and function. (Adapted from Pham et al. *Neurosurg Focus* 2011 [76] and He et al. *Neurosurg Focus* 2013 [91])

Gene	*Locus*	*Product*	*Genetic alteration*	*Normal function*	*Pathogenic impact in meningiomas*
Chromosome 22					
NF2	22q12.2	Merlin	Downregulation	Linkage of cell membrane proteins to the cytoskeleton	Early event in tumorigenesis [4, 77]
BAM22	22q12.2	Beta-adaptin	Downregulation	Endocytosis	Potential early event in tumorigenesis [107]
BCR	22q11	Bcr	Downregulation	Serine/threonine kinase, GTPase activator	Potentially involved in tumorigenesis [108]
TIMP3	22q12.3	Metalloproteinase inhibitor 3	Hypermethylation	Inhibits MMP-2 and MMP-9 activity	Associated with high grade tumors [109, 110]
Chromosome 1					
ALPL	1p36.1-p34	Alkaline phosphatase	Downregulation	Cell cycle control	Associated with high grade tumors and recurrence [78, 127, 128]
TP73	1p36.3	TP73	Hypermethylation	Blocks pro-apoptotic function	Associated with high grade tumors [110, 125, 126]
Chromosome 6					
HIST1H1C	6p21.1	Histone H1.2	Upregulation	Cell cycle	Associated with recurrence [71]
CTGF	6q23.2	Connective tissue growth factor	Downregulation	Growth factor	Associated with recurrence [71]
Chromosome 9					
CDKN2A	9p21.3	P16	Downregulation; Hypermethylation	Cell cycle control	Associated with high grade tumors [110, 122, 130, 131]
CDKN2B	9p21.3	P15	Downregulation; Hypermethylation	Cell cycle control	Associated with high grade tumors [122, 130, 131]
p14ARF	9p21.3	P14	Downregulation; Hypermethylation	Cell cycle control	Associated with high grade tumors [110, 122, 131, 133]
KLF4	9q31	Kruppel-like factor 4	Upregulation	Transcription factor which induces pluripotency	Associated with tumorigenesis of non-NF2 and secretory meningiomas [106, 167]
WNK2	9p22.31	WN kinase	Hypermethylation	Growth factor	Associated with tumorigenesis [166]
Chromosome 14					
NDRG2	14q11.2	NDRG2	Downregulation; Hypermethylation	Potentially involved in cell growth & apoptosis	Associated with high grade tumors and recurrence [145, 146]

Table 2. *(continued)*

MEG3	14q32	Noncoding RNA	Downregulation; Hypermethylation	Cell cycle	Linked to tumorigenesis & high grade tumors [143]
AKT1	14q32	Serine/threonine-protein kinase	Upregulation	Cell growth, Proliferation (activation PI3K pathway)	Associated with tumorigenesis of non-NF2 meningiomas [106, 144]
TMEM30B	14q	Transmembrane protein 30B	Downregulation	Cell cycle	Associated with tumor recurrence [71]
Chromosome 17					
STAT3	17q21.2	Signal transducer and activator of transcription 3	Upregulation	Transcription factor	Associated with high grade tumors [150, 151]
RPS6K	17q23	Ribosomal protein S6 kinase ($p70^{S6K}$)	Upregulation	Cell growth, Proliferation	Potentially involved in tumorigenesis [149]
IGF2BP1	17q21.32	RNA binding protein	Hypermethylation	Transcription factor	Associated with tumorigenesis [163, 165]
Chromosome 18					
DAL-1	18p11.32	4.1B	Downregulation	Links cell membrane proteins to cytoskeleton	Early event in tumorigenesis [158, 159] / associated with progression [161]
bcl-2	18q21.33	Bcl-2	Upregulation	Regulator of apoptosis	Associated with high grade tumors and recurrence [154-156]
Other chromosomes					
SMO	7q32.3	Smoothened, G protein-coupled receptor	Upregulation	Cell growth, proliferation (activation Hh pathway)	Associated with tumorigenesis of non-NF2 meningiomas [106, 144]
HOXA6 HOXA9	7p15.2	HOXA6, HOXA9	Comethylation	Transcription factor	Associated with tumorigenesis [163, 164]
TSLC1	11q23.2	CADM1	Downregulation	Cell adhesion	Associated with high grade tumors [168]
GSTP1	11q13	Glutathione S-transferase	Hypermethylation	Detoxification	Associated with high grade tumors [131, 132]
RB1	13q14.2	pRb	Hypermethylation	Cell cycle	Potentially involved in tumorigenesis [90, 131]
THBS1	15q15	Thrombospondin 1	Hypermethylation	Inhibition of angiogenesis	Associated with angiogenesis [110, 131]
TRAF7	16p13.3	TNF receptor-associated factor 7	Several mutations	Proapoptotic E3 ubiquitin ligase	Associated with tumorigenesis of non-NF2 meningiomas [106, 167]
CDH1	16q22.1	E-cadherin	Downregulation	Cell adhesion	Associated with high grade tumors, recurrence and invasion [169-171]
TIMP1	Xp11.3-p11.23	Metalloproteinase inhibitor 1	Downregulation	Inhibits MMP-9 activity	Tumor invasion [112, 113]

2.5. Altered signaling pathways in meningiomas

At present, it is well known that most of the above genetic alterations have an impact on one or more signaling pathways which are recurrently involved in cancer. In this section we will review the most relevant and frequently altered signaling pathways in meningioma (Figure 2).

2.5.1. The pRB/p53 pathways and its impact on cell cycle dysregulation

Genetic alterations of the *p16*^{INK4a}, *p15*^{INK4b}, and *p14*^{ARF} tumor suppressor genes are commonly observed in meningiomas, particularly among the anaplastic subtype, leading to dysregulation of the pRB- and p53-dependent pathways [3, 122, 130, 133]. pRB has a central role in the inhibition of cell cycle progression at the G1/S-phase checkpoint. Briefly, pRB functions through its binding (and inhibition) to the E2F transcription factor. Once cyclin D expression is upregulated (e.g. under mitogenic stimuli) it binds to either Cdk4 or Cdk6, and phosphorylates pRB; pRB phosphorylation induces the release of the active E2F factor, leading to the transcription of genes which are critical to entry into S-phase. *p16*^{INK4a} and *p15*^{INK4b} prevent S-phase entry by inhibiting the Cdk4/cyclin D complex [77]. In turn, the p53 pathway acts as a feedback inhibitor of the pRB pathway, inducing cell cycle arrest, DNA repair, and apoptosis in case of aberrant pRB pathway activation. The two pathways are connected via p14^{ARF}. The release of the E2F transcription factor, following phosphorylation of pRB, also induces transcription of *p14*^{ARF}, which promotes p53 activity through negative regulation of the proto-oncogene MDM2 (murine double minute 2 protein) [3]. Dysregulation of these two pathways in higher-grade meningiomas is frequently associated with loss of p16^{INK4a}, p15^{INK4b} and p14^{ARF} and increased cell proliferation, together with tumor progression [122, 130, 133]. In addition, accumulating evidences indicate that loss of function of *RB1* by hypermethylation [90, 131], overexpression of the *MDM2* gene and its protein [59, 129, 172], and loss of expression of *MEG3* (anti-proliferative tumor suppressor that stimulates the activation of p53 mediated by transcriptional effect) [143] in higher grade meningiomas, might further contribute to dysregulation of both cell cycle-associated pathways in meningioma progression.

2.5.2. Growth factors and autocrine loops

Multiple studies have demonstrated enhanced expression of several growth factors, as well as activation of autocrine loops, considered to be relevant in the pathogenesis of meningiomas. Meningioma growth factors consist of a large and diverse family of proteins that

act as extra and intracellular signals inducing tumor growth, cell migration, and angiogenesis, many of which exert their effect via the MAPK and PI3K/Akt signaling pathways [173]. Among others, the platelet-derived growth factor BB (PDGF-BB) and its PDGFR-β receptor are frequently overexpressed in meningiomas, typically at greater levels among high *vs.* low grade tumors [174, 175]. Evidences indicate that PDGF-BB stimulates meningioma cell proliferation via an autocrine and/or paracrine loop, treatment with anti-PDGF-BB agents showing inhibition of meningioma cell growth [175, 176]. In addition to PDGF-BB and PDGFR-β, the epidermal growth factor receptor (EGFR), as well as both the EGF and the transforming growth factor-alpha (TGF-α) ligands, are all widely expressed on meningioma cells, representing another potential autocrine loop that may stimulate meningioma cell proliferation [177-179]. Other growth factors reported in meningiomas include members of the insulin-like growth factor (IGF) system, such as IGF2 and several IGF-binding proteins (e.g. IGFBP2); in this regard, higher levels of IGF2 have been associated with a greater invasiveness and/or tumor progression [180-182]. Interestingly, Lallemand et al. [94] reported that merlin regulates cell contact-mediated inhibition of proliferation by limiting the delivery of several growth factor receptors (e.g. ErbB2, ErbB3, IGF1R and PDGFR) at the plasma membrane of primary Schwann cells; such decreased delivery of growth factor receptors would inhibit the activity of the downstream mitogenic signaling pathways.

The vascular endothelial growth factor A (VEGFA) and its VEGFR-1 receptor have been associated with regulation of the development of new blood vessels and peritumoral edema in brain tumors, a common feature in meningioma patients [77, 173]. In this regard, meningiomas express both VEGF and VEGFR, and the severity of peritumoral edema frequently correlates with VEGF expression [55, 56, 183-187]. In addition, an association between VEGF expression and both tumor vascularization [55, 183, 185, 187] and meningioma grade [57, 188] has been also reported, but not definitively confirmed. Despite all the above, the precise mechanisms that regulate VEGF expression in meningiomas remain unknown. In human cells, VEGF is mainly regulated by the hypoxia inducible factor-1 (HIF-1) transcription factor; in meningiomas, HIF-1 expression correlates with VEGF expression and the degree of peritumoral edema [189, 190]. In addition, upregulation of VEGF expression can also be induced by other growth factors such as EGF and PDGF, suggesting that both growth factors and hypoxia stimulation may contribute to VEGF control [190], further studies being required in this regard.

Other growth factors that have been associated with the pathogenesis of meningiomas include: 1) the stromal cell derived factor 1 (SDF1) CXC chemokine and its CXCR4 receptor, which might exert its mitogenic effects through the MAPK pathway [191]; 2) the bone morphogenic proteins (BMPs) and their receptors (BMPR), which are associated with Smad 1

signaling [192], and; 3) the fibroblast growth factor (FGF) and its FGFR3 receptor, which are activated by the PI3K/Akt pathway [193]. In contrast, TGF-β and its (type I and type II) receptors (TGF-βRI and TGF-βRII) may act as potential inhibitors of meningioma growth/proliferation through the Smad 2/3 apoptotic pathway [194, 195], although the role of TGF-β in meningioma tumorigenesis remains to be fully established.

2.5.2.1. The MAPK and PI3K/Akt signaling pathways

The mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway are both involved in multiple cellular processes (e.g. differentiation, growth, and apoptosis) associated with the pathogenesis of meningiomas, particularly with those tumors showing deregulated cell proliferation [77, 173]. MAPKs are intracellular serine/threonine-specific protein kinases which are activated by extracellular stimuli (e.g., mitogen signals), leading to the sequential activation of a kinase cascade triggered by the Ras/Raf-1/MEK-1/MAPK/ERK pathway. Upstream activation of this pathway ultimately leads to phosphorylation/activation of transcription factors in the cell nucleus [195]. Evidences for the activation of the MAPK pathway in meningiomas have been recurrently reported [174, 196]. Thus, Johnson et al. [174] reported that both PDGF-BB and cerebrospinal fluid (which contains multiple meningioma cell growth factors) stimulate the proliferation of both WHO grade I primary meningioma cells and cultured meningothelial cells, mediated at least in part, through activation of the MAPK/ERK pathway [174]; furthermore, treatment with MAPK inhibitors has been shown to induce progressive growth inhibition of meningioma cells in association with reduced MAPK phosphorylation/activity [174].

PI3Ks are a family of intracellular signal transducer enzymes that phosphorylate inositol phospholipids. Activation of PI3K results in phosphorylation/activation of PKB/Akt and subsequently $p70^{S6K}$, which are key elements of the cell growth promoting effects of this pathway [195]. Johnson et al. also reported that Akt/PKB and $p70^{S6K}$ are expressed and activated in meningiomas, and transduce growth signals of mitogens such as PDGF-BB [197] and FGF [193]; in line with their results, administration of PI3K inhibitors blocks PDGF-BB stimulation and decreases Akt and $p70^{S6K}$ phosphorylation in meningioma cells [197]. Interestingly, Mawrin et al. [196] have found higher levels of phospho-Akt/PKB in association with lower levels of activation of MAPK in both anaplastic and atypical *vs.* benign/grade I meningiomas, such a signaling profile being associated with tumor recurrence. Moreover, *in vitro* studies revealed decreased meningioma cell growth in the presence of a PI3K blocker, in the absence of apoptosis, whereas inhibition of MAPK resulted in cell death through apoptosis

[196]; based on these findings it could be hypothesized that PI3K/Akt activation is associated with aggressive growth in malignant meningiomas, whereas MAPK activation appears to be involved with both meningioma cell proliferation and apoptosis [196].

2.5.2.2. PLCγ-PKC and calcium signaling

Tyrosine kinase receptors such as EGFR and PDGFR also activate (e.g. phosphorylate) phospholipase C-γ1 (PLC-γ1), leading to hydrolysis of PIP2 (phosphatidylinositol 4,5-biphosphate) into two intracellular active second messengers: IP3 (inositol 1,4,5-triphosphate) and 1,2-DAG (1,2-diacylglycerol). DAG activates protein kinase C (PKC), which enters the nucleus and activates transcription factors, resulting in cell proliferation and inhibition of apoptosis [195]. Johnson et al. [179] reported that activation of the EGFR kinase on meningioma cells further activates PLC-γ1 and increases its catalytic activity, leading to another mechanism that promotes meningioma cell growth; additional evidences indicate that PLCγ expression does not differ significantly between meningiomas of different histopathology grades [196]. In turn, IP3 mediates calcium release from intracellular calcium stores resulting in increased free cytosolic calcium [173]. Interestingly, calcium channel antagonists can block *in vitro* primary meningioma cell growth after stimulation with EGF and PDGF [198], as well as *in vivo* meningioma growth in a subcutaneous meningioma mouse model [199]. However, the mechanism of calcium channel antagonist interruption of IP3-mediated intracellular calcium pathways in meningiomas, remains unclear and deserves further investigation.

2.5.2.3. Cyclooxygenase-2 signaling

The phospholipase A2 (PLA2)-cyclooxygenase (COX) signaling pathway has also been recently investigated in meningiomas [195]. COX-2 is an enzyme that serves as the rate-limiting step for the synthesis of prostaglandins from arachidonic acid. Prostaglandins, such as PGE2, are mediators of several critical cellular processes involved, among others, in cell growth, proliferation, adhesion, angiogenesis, suppression of apoptosis, and inflammation [77]. Normally, the cytoplasmic levels of arachidonic acid are relatively low, which limits the production of prostaglandins. However, altered levels of arachidonic acid and COX-2 overexpression have been both detected in many tumors, where they are associated with cancer growth and progression, possibly driven by signaling pathways such as the MAPK pathway. In fact, Ras activation of MAPK, induced by growth factors such as EGFR, stimulates *COX-2* transcription and increases the cytosolic PLA2 hydrolysis of membrane phospholipids,

releasing arachidonic acid at higher concentrations [195]. Of note, high levels of arachidonic acid, increased prostaglandin production [200], as well as overexpression of COX-2 [201], have also been reported in meningiomas. In addition, COX-2 expression has been correlated with a greater degree of invasiveness to the brain or the adjacent soft tissues [57], tumor recurrence [59] and higher MIB-1 labeling index [202] and VEGF levels [57, 202], suggesting it may play an important role in the development and growth of meningiomas.

2.5.2.4. The mTOR signaling pathway

Recent studies have found the mammalian target of rapamycin (mTOR), a critical modulator of cell growth and proliferation, to be also involved in the signaling pathways associated with meningioma tumorigenesis. mTOR is a protein kinase that may be expressed in two distinct complexes (mTORC1 and mTORC2). mTORC1 regulates cell growth by promoting increased translation and protein synthesis through phosphorylation of the effector proteins $p70^{S6K}$ and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1); in turn, mTORC2 directly phosphorylates Akt, a step required for its full activation [84]. More recently, merlin has also been identified as a negative regulator of mTORC1, at the same time that activation of mTORC1 has been associated with meningioma growth [203]. In this regard, James et al. [203] have demonstrated that mTORC1 levels are elevated in tumors derived from patients with NF2 disease and in fibroblasts from an NF2-deficient mouse model. Although the exact mechanism still remains unclear, merlin inhibits mTORC1 through a novel pathway, which is independent from the previously established activators of the mTORC1 pathway (e.g. PI3K and MAPK) [203]. Thus, inhibition of the mTORC1 pathway (e.g. through rapamycin) represents a promising route for targeted therapeutics in meningiomas as also supported by the observation of suppression of meningioma growth by mTORC1 inhibitors in mouse models [204].

In contrast to its effects on mTORC1, merlin positively regulates the kinase activity of mTORC2, downstream phosphorylation of mTORC2 substrates, including Akt, being reduced upon acute merlin deficiency in cells [205]. However, the attenuated mTORC2 signaling profiles in response to merlin loss could not be detected in NF2-deficient meningiomas [205].

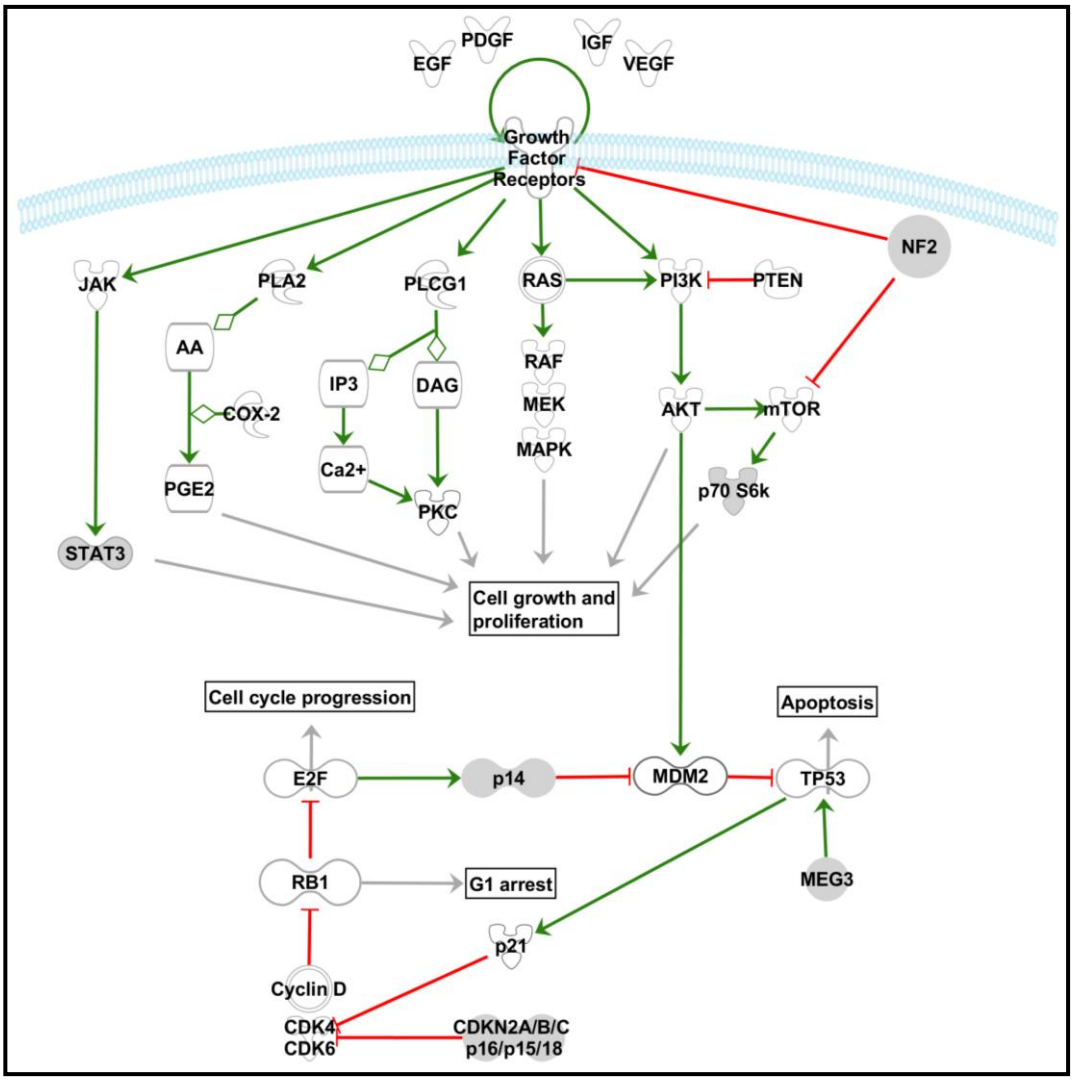

Figure 2. Schematic diagram illustrating the key elements of some of the most relevant signaling pathways involved in the pathogenesis of meningiomas. The Ras-Raf-MEK-MAPK/ERK, PI3K-Akt/PKB, PLCγ1-PKC, PLA2-COX, JAK-STAT3 and mTOR signaling pathways are represented in the upper part of the scheme, while the relationships between the pRb and p53 cell cycle pathways are illustrated in the lower part of the scheme. The pathway scheme displayed was generated with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA).

2.5.3. The WNT/Beta-Catenin pathway

The wingless (wnt)/β-catenin pathway has also been implicated in meningioma progression. In this regard, early studies based on microarray gene expression profiling identified increased expression of several genes associated with the Wnt pathway, such as the *CTNNB1*, *CDK5R1*, *ENC1* and *CCND1* genes [180]. Subsequently, Pecina-Slaus et al. reported LOH of the E-cadherin (*CDH1*) [206] and the adenomatous polyposis coli (*APC*) genes [207] in about one-third and half of the cases, respectively, both tumor suppressor genes being directly involved in the Wnt/β-catenin pathway. Downregulation of E-cadherin (protein) expression in clinically aggressive and invasive meningiomas had already been described [169-171] in

association with upregulation and nuclear/perinuclear localization of β-catenin [206, 208], suggesting an important role for E-cadherin in meningioma tumorigenesis. Interestingly, Zhou et al. [209] suggested a model in which active merlin would inhibit Wnt/β-catenin signaling and maintain β-catenin and N-cadherin complexed at the plasma membrane; loss of merlin in some tumors, would then lead to loss of contact inhibition and activation of the Wnt/β-catenin signaling (and consequently also, of the PDGFR/Src and Rac/PAK pathways), translocation of β-catenin to the nucleus and expression of intracellular growth-associated proteins such as c-myc and cyclin D1.

The *BCR* gene which is encoded at chromosome 22q11, represents a tumor suppressor candidate involved in the pathogenesis of meningiomas. In this regard, low expression of BCR is typically detected in meningiomas with LOH at chromosome 22q [108], and it has been shown to be a negative regulator of the Wnt pathway [210]. Interestingly, Pérez-Magán et al. [72], using gene expression profiling, recently reported a genetic signature of advanced and recurrent meningiomas, which included aberrant expression of genes of the Wnt pathway; thus, these authors found downregulation of *SFRP1*, a gene from the family of secreted frizzled-related proteins (SFRPs) which are able to downregulate Wnt signaling, in recurrent and atypical meningiomas.

2.5.4. The Notch Pathway

The Notch signaling pathway is involved in extracellular-to-intracellular signaling mediated through the Notch1-4 transmembrane proteins. Ligand proteins bind to the extracellular portion of the Notch proteins, resulting in proteolytic cleavage and release of the intracellular portion; such intracellular Notch protein fragments translocate to the nucleus and initiate the expression of the Hairy/Enhancer of Split (HES) family of transcriptional regulators [77, 173]. Cuevas et al. [211] comparatively analyzed the gene expression profiles of normal/reactive meninges and meningiomas of all histopathological grades and demonstrated the potential involvement of the Notch signaling pathway in meningiomas. Thus, HES1 expression was increased in all meningioma grades and HES1 expression correlated with increased expression of Notch1, Notch2, and the Jagged ligand; in contrast, transducin-like enhancer of split (TLE) 2 and TLE3, two co-repressors that modulate HES1 activity, were specifically upregulated in malignant meningiomas. Furthermore, deregulation of notch in meningiomas results in tetraploidy and chromosomal instability [212], further studies being required to elucidate the precise mechanism by which abnormal notch activation induces such genetic changes in meningiomas.

2.5.5. The Hedgehog (Hh) signaling pathway

The Hh signaling pathway is a critical regulator of both development during embryogenesis and adult homeostatic processes [76, 173]. When Hh binds its patched (PTCH) receptor, the Smoothened (SMO) transmembrane protein is activated and initiates a signaling cascade that results in the activation of GLI transcription factors (e.g. GLI1 and GLI2 growth activators) and subsequent transcription of genes implicated in cell growth, proliferation, angiogenesis, matrix remodeling, and stem cell homeostasis [77, 173]. Recently, Laurendeau et al. [213] have analyzed the mRNA expression patterns of 32 Hh pathway-related genes in 36 meningiomas and found increased levels of 16 genes involved in the activation of the Hh pathway (e.g. *SMO*, *GLI1*, *GLI2*, *GLIS2*, *FOXM1, IGF2* and *SPP1)* and cell growth, together with decreased expression of 7 genes involved in the inhibition of the Hh pathway (e.g. the *PTCH1* tumor suppressor*)*; some of these genes further showed different expression profiles among tumors of different histopathological grades, suggesting distinctly altered profiles early during tumorigenesis *vs.* progression to more aggressive tumor lesions. Interestingly, recent reports have identified *SMO* mutations in meningiomas lacking *NF2* mutations [106, 144], which further supports the potentially relevant role of this pathway in the development of at least some meningiomas.

2.6. Cytogenetic subgroups of meningiomas and tumor progression

For the identification of genetic alterations at individual chromosomes, conventional cytogenetic karyotyping was initially used [214]. More recently, molecular cytogenetic methods such as fluorescence *in situ* hybridization (FISH) for specific targeted chromosomal regions [215], emerged as a useful tool for the detection of chromosomal alterations at the single cell level in meningiomas [63, 64, 117, 120, 216-219]. By FISH, various different types of cytogenetic alterations may be identified such as aneusomy (e.g. numerical chromosomal alterations), duplication, amplification, deletion, and translocation of specific chromosomal regions targeted by the FISH probes [215]. Subsequently, FISH-based tests such as comparative genomic hybridization (CGH) and array CGH were used [63, 129, 147, 148, 219-222] for higher resolution definition of complex karyotypes and global scanning of recurrent genomic imbalances in tumor cells [214].

Based on the results obtained through the usage of the above highlighted techniques, the first cytogenetic classifications and cytogenetic models of progression of meningiomas were proposed [62, 78, 129, 134, 223]. Thus, Weber et al. [129] used CGH to screen for

chromosomal imbalances in meningiomas from different grades and they proposed a model of genomic alterations associated with meningioma progression (Figure 3A). Later on, Ketter et al. [62] and Zang et al. [78], subdivided meningiomas into four subgroups based on their cytogenetic findings: Group 0, included meningiomas with a normal diploid chromosomal set; Group 1, consisted of tumors with monosomy 22 as the sole cytogenetic alteration; Group 2, was composed of tumors showing markedly hypodiploidy with loss of additional autosomes, and finally; Group 3 included meningiomas with deletions of the short arm of chromosome 1, in association with other chromosomal aberrations including loss of chromosome 22. Furthermore, these authors also applied oncogenetic tree mixtures to estimate typical pathogenetic routes as regards the sequence of accumulation of somatic chromosomal changes in tumor cells, based on which, a genetic progression score (GPS) was generated. Through the GPS, meningiomas were categorized into three groups of increasingly higher genetic complexity associated with tumor/cytogenetic progression: GPS Group 0, characterized by absence of loss of chromosome 22; GPS Group 1, mainly consisted of tumors with monosomy 22, and; GPS Group 2, composed of tumors carrying at least loss of one chromosome 22 and loss of chromosome 1p [223] (Figure 3B). Subsequently, Sayagues et al. [224] investigated the intratumoral cytogenetic patterns of clonal evolution at individual tumors and used such intratumoral cytogenetic profiles to establish tumor progression pathways in meningiomas. Thus, complete or partial loss of either chromosome 22, a sex chromosome (i.e. Y in males and X in females), del(1p) or less frequently, monosomy 14/14q$^-$ alone or in combination with other chromosomal losses (e.g., monosomy 10/10q$^-$ and 18/18q$^-$), would frequently represent the earliest detectable cytogenetic event in meningioma tumor cells (Figure 3C). Interestingly, despite the clear association observed between a more advanced tumor grade and a higher number of tumor cell clones and complex karyotypes, authors found that the pathways of intratumoral clonal evolution observed in benign meningiomas were markedly different from those most frequently observed in atypical/anaplastic tumors; altogether, these results suggest that the latter tumors might not always represent a more advanced stage of histologically benign meningiomas, but they could more likely represent stages of distinct clonal evolution pathways.

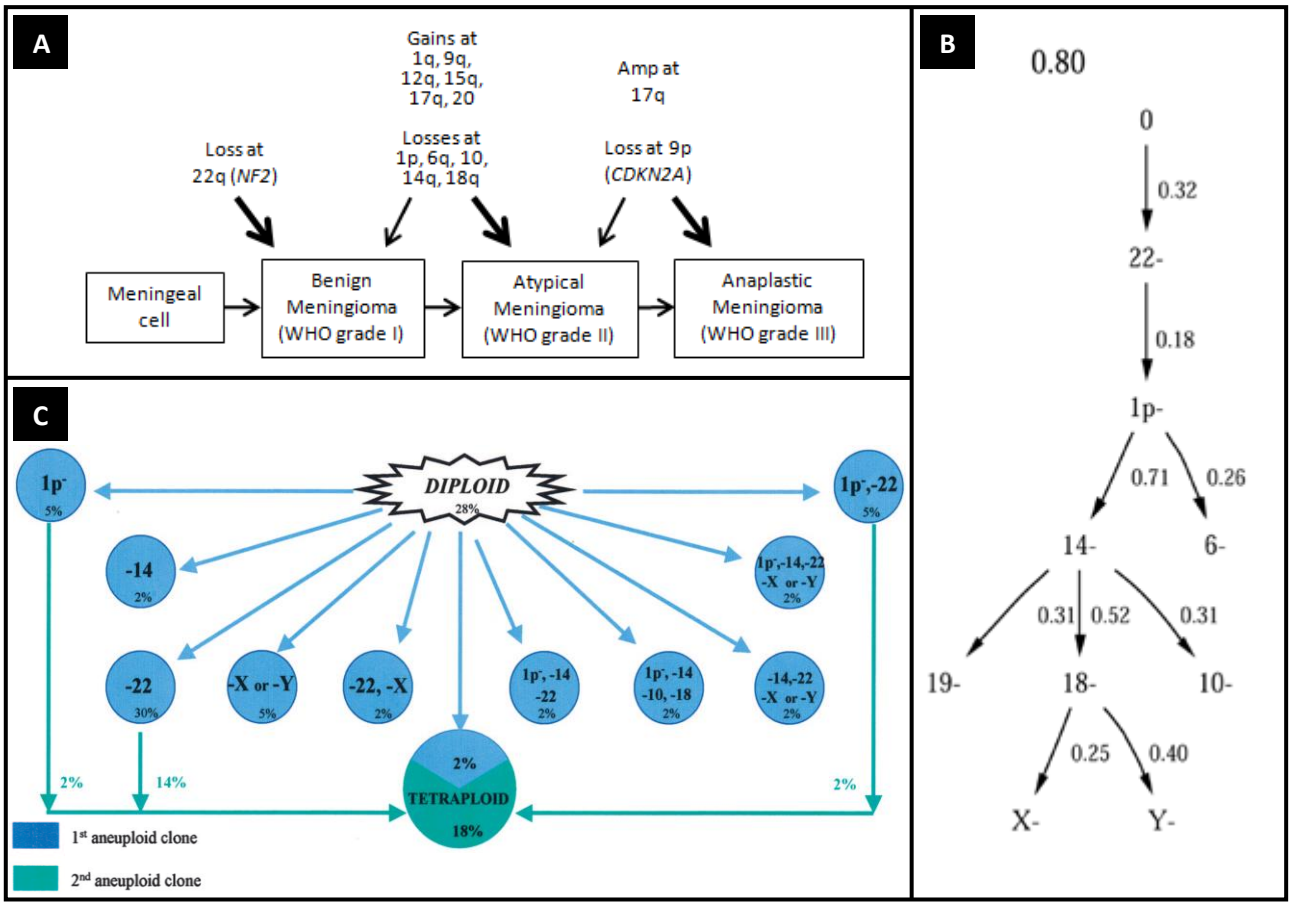

Figure 3. Examples of three models of cytogenetic alterations proposed to reflect cytogenetic progression of meningiomas, according to (A) Weber et al. [129], (B) Ketter et al. [62], and (C) Sayagues et al. [224]. (A) In this model, progression from grade I to grade III is proposed to occur in parallel to the acquisition of specific chromosomal gains and losses at frequencies of more than 30% of cases; nevertheless, chromosomal changes may already have occurred in a lower grade in a smaller percentage of tumors (thin arrows are pointing toward the lower tumor grade to illustrate this fact). **(B)** Oncogenetic tree mixture model for the acquisition of chromosomal alterations in the development of meningiomas (edges are labeled with conditional probabilities); the first two critical steps in the progression model correspond to monosomy 22, followed by loss of the short arm of chromosome 1. **(C)** Hypothetical intratumoral aneuploidization pathways defined on the basis of the patterns of clonal evolution observed for 11 chromosomes analyzed; percentage values correspond to the frequency of cases with a tumor cell clone displaying a specific cytogenetic pattern. (Modified from Weber et al. *Proc Natl Acad Sci U S A* 1997 [129], Ketter et al. *J Neurosurg* 2001 [62] and, Sayagues et al. *J Mol Diagn* 2004 [224])

In the last decade, high-throughput technologies and next generation sequencing have also been developed and applied to the study of meningiomas. Through these technologies, analysis of the whole-genome can be obtained in a fast and accurate way, providing a new discovery tool for the identification of new candidate genes and pathways associated with the development, progression, and invasiveness of meningiomas [225]. Whole-genome microarray technologies were first applied to the study of the gene expression profile (GEP) of tumor cells [225]. In a pioneering study in meningiomas, Watson et al. [181] reported tumor GEP to be associated with the WHO grades. Subsequently, additional microarray-based studies have

further investigated the potential existence of meningioma-specific genes and of genes associated with the WHO grade [180, 181, 226, 227] or the main histopathological subtypes of grade I meningiomas [226, 228]. For example, Wrobel et al. [180] identified 37 genes with decreased expression and 27 genes with upregulated expression in atypical and anaplastic *vs.* benign meningiomas, and Fèvre-Montange et al. [226] used unsupervised hierarchical clustering to classify meningiomas into three groups that closely mimicked the three WHO grades, with higher proliferation indexes and/or frequencies of recurrence being included in the atypical group; in addition, they also identified among benign meningiomas, genes with signatures that were highly specific for fibroblastic (*FBLN1*, *Tenascin C* and *MMP2* encoding extracellular matrix proteins) and meningothelial (*MLPH*, *DEFB1* and *FAT3*) tumors, suggesting that different mechanisms might be involved in tumorigenesis in such subtypes. Other microarray-based studies identified some of the signaling pathways specifically activated in meningiomas [72, 180, 229-231], such as the Wnt pathway [72, 180]. In a similar way, GEP of meningioma cells have also proven to be associated with tumor localization (e.g. spinal *vs.* intracranial tumors) [232], patient gender [233], and the clinically relevant cytogenetic subgroups of meningiomas (i.e. cases with normal karyotype, isolated monosomy 22/del(22q) and complex karyotype) [73].

Most interestingly, GEP of meningiomas have shown the existence of heterogeneous profiles associated with different tumor behavior even within the same WHO grade; thus, tumor cells from aggressive and/or invasive meningiomas appear to display unique GEP [72, 74, 145, 226], typically associated with high-proliferative gene expression signatures [234], and high-risk of recurrence [72-74] (Figure 4). In this regard, Carvalho et al. [234] showed that meningiomas fall into two main molecular subgroups designated as 'low-proliferative' and 'high-proliferative' meningiomas, according to their different GEP and median MIB-1 labeling indices; of note, the latter group also showed greater frequency of copy number alterations. In addition, authors found that the major molecular mechanisms that distinguish between the two groups were: gain of cell proliferation markers and loss of components of the TGF-β signaling pathway. Similarly, Perez-Magan et al. [72] identified a 49-gene signature of meningioma aggressiveness that characterizes histologically benign meningiomas which may recur; such signature classified the tumors into 2 groups with different clinical and pathological behaviors, and was composed of genes involved in the cell cycle as well as other (e.g. Wnt and TGF-β) signaling pathways. Overall, advanced/recurrent tumor samples showed global downregulation of gene expression *vs.* benign/primary tumors, which according to the authors could be associated with distinct epigenetic profiles together with more extended losses of

specific chromosomal regions, both events therefore potentially playing an important role in progression and recurrence of meningiomas.

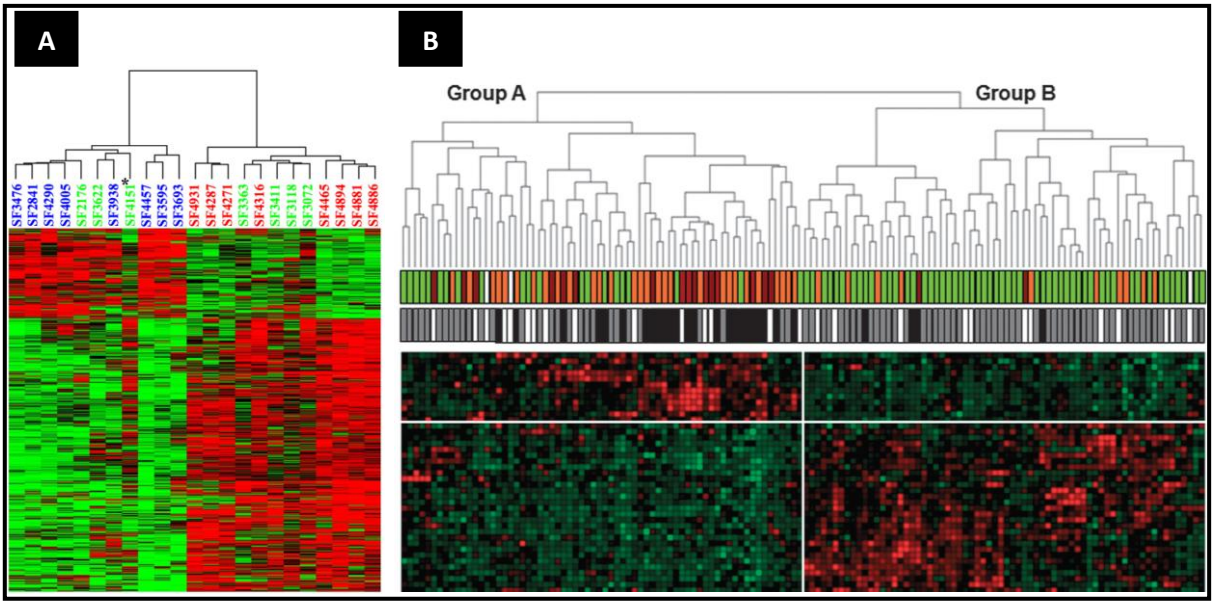

Figure 4. Illustrating examples of gene expression profiling subgroups of meningiomas as defined by (A) Carvalho et al. [234] and by (B) Perez-Magan et al. [72]. Unsupervised hierarchical clustering of gene expression data derived from meningiomas classified according to the WHO tumor grade show presence of two major molecular subgroups defined according to their proliferative/aggressive *vs.* indolent profile. Expression values are color-coded as red for higher and green for lower expression. Each column represents one case whereas each row displays the expression of an individual gene. **(A)** Meningioma cases are coded as red (grade I), green (grade II), and blue (grade III); all benign meningiomas fall into the 'low-proliferative' group and all malignant grade III tumors fall into the 'high-proliferative' group, whereas atypical/grade II meningiomas are distributed into both groups. **(B)** Meningioma cases are coded as green (grade I), orange (grade II), dark red (grade III), black (recurrent), and gray (nonrecurrent) bars; most WHO grade II/III and grade I meningiomas corresponded to the more aggressive (A) and the less aggressive (B) groups, respectively. (Adapted from Carvalho et al. *Mol Cancer* 2007 [234] and Perez-Magan et al. *J Neuropathol Exp Neurol* 2012 [72])

In parallel to GEP arrays, single nucleotide polymorphism (SNP)-arrays have also been used in recent years for genome-wide characterization of meningiomas through the interrogation of hundreds of thousands of SNPs scattered through the human genome [76]. Based on SNP-arrays, a detailed description of high-resolution genome-wide CNV and LOH alterations has been obtained in meningiomas and used for their genetic subclassification [75, 118, 130, 235]. Thus, Lee et al. [75] described 5 'classes' of meningiomas based on gene expression analyses that showed a high correlation with their copy number alteration profile, as well as the tumor recurrent status and histopathology (Figure 5). Similarly, Tabernero et al. [118] suggested that meningiomas could be classified into 3 subgroups based on their overall SNP-array profiles: diploid cases, meningiomas with a single chromosomal change [e.g. monosomy 22/22q$^-$] and tumors with complex karyotypes including ≥2 altered chromosomes (Figure 6).

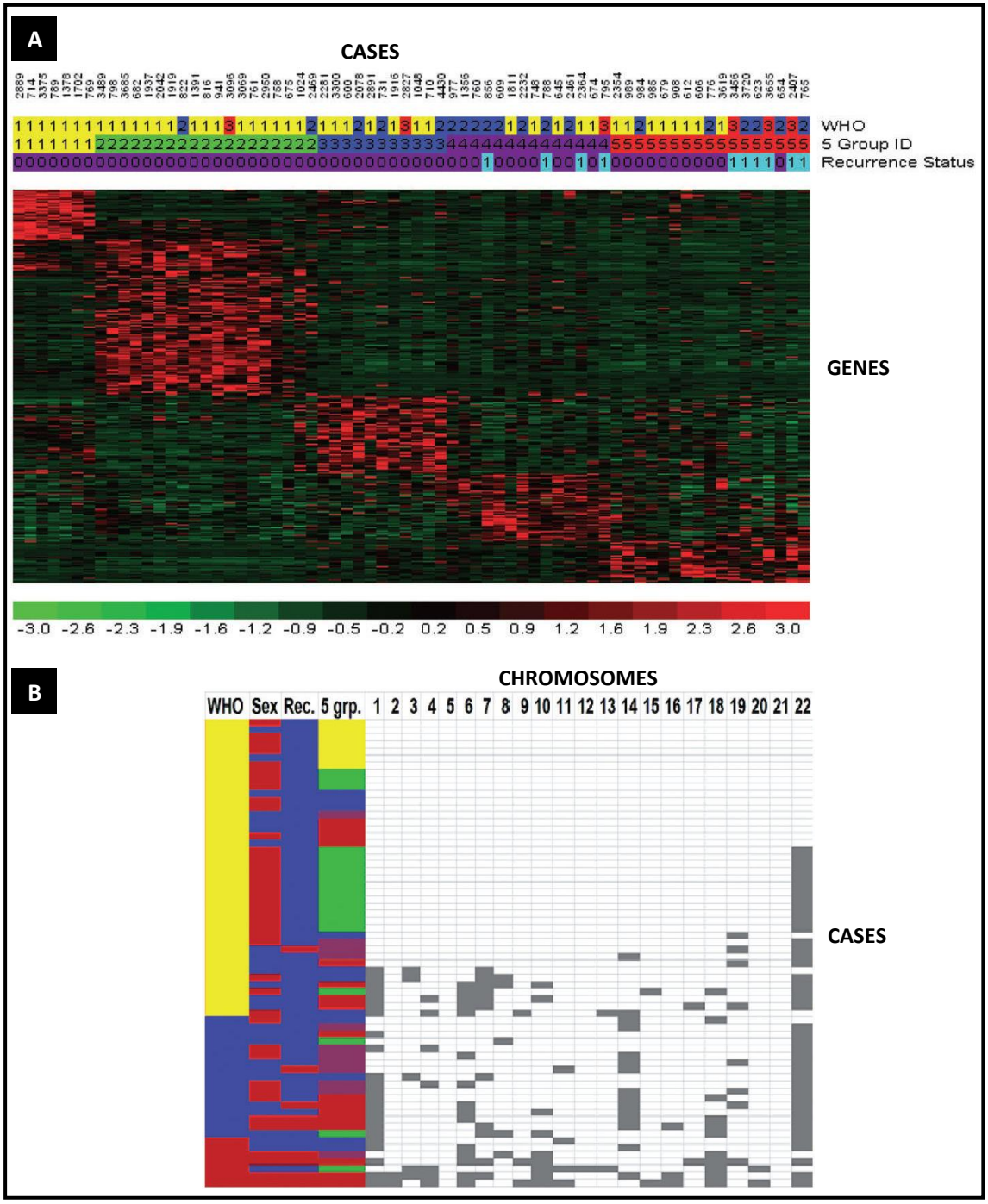

Figure 5. Gene expression profile-based subgroups of meningiomas as defined by Lee et al. [75]. The GEP classifier identified 5 tumor groups based on 302 genes. Correlation between the GEP of tumor cells (classified into the 5 patients groups) and the WHO grade, patient sex, and tumor recurrence status (panel A), or the distribution of chromosome losses (panel B, gray squares) are shown. In panel A, each column represents a tumor and each row corresponds to a different gene; in panel B, each row corresponds to a different patient, while columns represent different chromosomes and other clinical variables. Color codes: WHO grade I (yellow), grade II (blue) and grade III (red); meningioma GEP groups: 1 (yellow), 2 (green), 3 (blue), 4 (purple) and 5 (red); recurrence status codes: newly-diagnosed meningioma (blue or codified value = 0) *vs.* recurrent meningioma (red or codified value = 1); sex codes: males (blue) and females (red). (Modified from Lee et al. *Brain Pathol* 2010 [75])

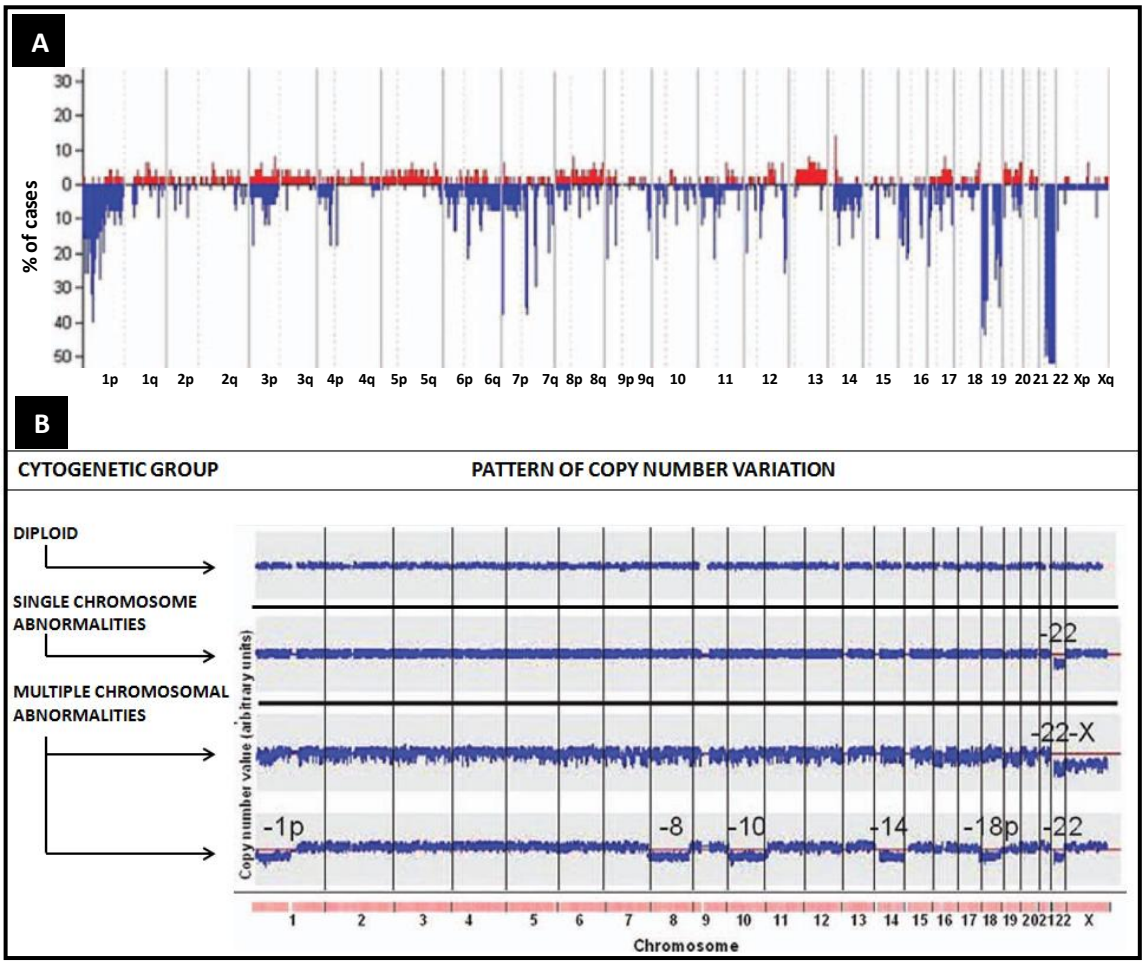

Figure 6. Frequency and extent of copy number variation (CNV) alterations observed by SNP-arrays for individual chromosomes in meningiomas, and illustrating examples of the three major CNV profiles described by Tabernero et al. [118]. (A) The overall frequency of gains and losses (*y*-axis) identified for the 23 human chromosomes are delineated by vertical lines (red and blue lines, respectively) for each individual loci analyzed within each chromosome (*x*-axis). **(B)** CNV profiles of four representative cases of the three major cytogenetic groups of meningiomas: diploid tumors, meningiomas with only one altered chromosome (e.g. monosomy 22/22q$^-$) and tumors with multiple chromosomal changes; the red line indicates where the hybridization signal would fit a normal diploid CN pattern. (Modified from Tabernero et al. *Genes Chromosomes Cancer* 2012 [118])

In addition, the latest studies have been applying next-generation sequencing technologies to identify new genetic alterations and/or new genes leading to meningioma development [236]. Some of the genes emphasized so far include the *AKT1* (recurrent mutation: E17K), *SMO* (L412F and W535L mutations), *KLF4* (recurrent mutation: K409Q) and *TRAF7* (several mutations mapped at the WD40 domains) genes [106, 144, 167]. Most interestingly, such mutations were shown to correlate with specific clinical characteristics (e.g. tumor localization and histopathological subgroups) as well as with a subset of meningiomas lacking *NF2* mutations, bringing some new insights to non-*NF2* mutated tumors [106, 144, 167].

Despite all the above, it should be noted that at present the cytogenetic features of meningiomas have not been incorporated into a well-accepted and widely used classification of meningiomas, as complementary information to that provided by tumor histopathology and grade.

3. TUMOR MICROENVIRONMENT IN MENINGIOMAS

Tumor development and growth typically requires an appropriate microenvironment, in addition to those oncogenic genetic/molecular alterations that had targeted the tumor cells. Such tumor microenvironment consists of a complex network of distinct cell types and extracellular matrix components, in which neoplastic cells interact with fibroblasts, vascular endothelial cells, a variety of infiltrating immune cells (including a network of cytokines and chemokines released by these cells) and extracellular matrix proteins and other components. Although tumor development and growth largely depend on an adequate tumor microenvironment, the tumor cells per se also induce significant changes in the tissue where they home and grow [237]. In this regard, it should be noted that, at the same time that oncogenic signals and genetic changes occur in a stepwise manner in the growing tumor, the microenvironment also changes over the course of the disease from the onset of the tumor to cancer progression, thereby emphasizing the relevance of the bidirectional communication between the tumor cells and their microenvironment [238].

Immune cells present in the tumor typically include T lymphocytes, natural killer (NK) cells, macrophages, dendritic cells, polymorphonuclear leukocytes and occasional B cells [237, 239]. Of note, infiltration by immune cells is a hallmark of virtually every tumor [238], and it is frequently associated with the tumor behavior and patient outcome [239]. In this regard, while multiple reports in the literature have linked the presence of inflammatory infiltrates in human tumors with an improved prognosis and a better patient outcome [239-242], many others have found no significant correlation or they have even linked immune cell infiltration with a poorer prognosis. Such apparent discrepancy may be due to the type and functional state of immune cells infiltrating the tumor [239, 243-245]. In fact, the different types of infiltrating immune cell populations vary not only according to the type of cancer, but also from patient to patient within the same type of tumor; these observations suggests that different immune cell microenvironments may have distinct effects/roles in tumor control and progression [239]. In addition, the same immune cells present in the tumor microenvironment modulate their anti- or pro-tumoral functions, being able to play dual roles with potential to either suppress or

promote malignancy (Figure 7) [246]. Usually, the latter predominates as the tumor cells acquire mechanisms for 'immune evasion'. The tumor not only manages to escape from the host immune system, but it also develops a phenotype capable of manipulating immune cells (e.g. via secretion of chemokines and cytokines), and modifying their function to create a microenvironment that would favor tumor progression [247]. To date, many mechanisms of immune evasion by tumor cells have been identified (Table 3), including inhibition of immune cell functions or apoptosis of anti-tumor effector cells, and further production of growth and angiogenic factors that stimulate tissue repair and consequently also, tumor growth [237].

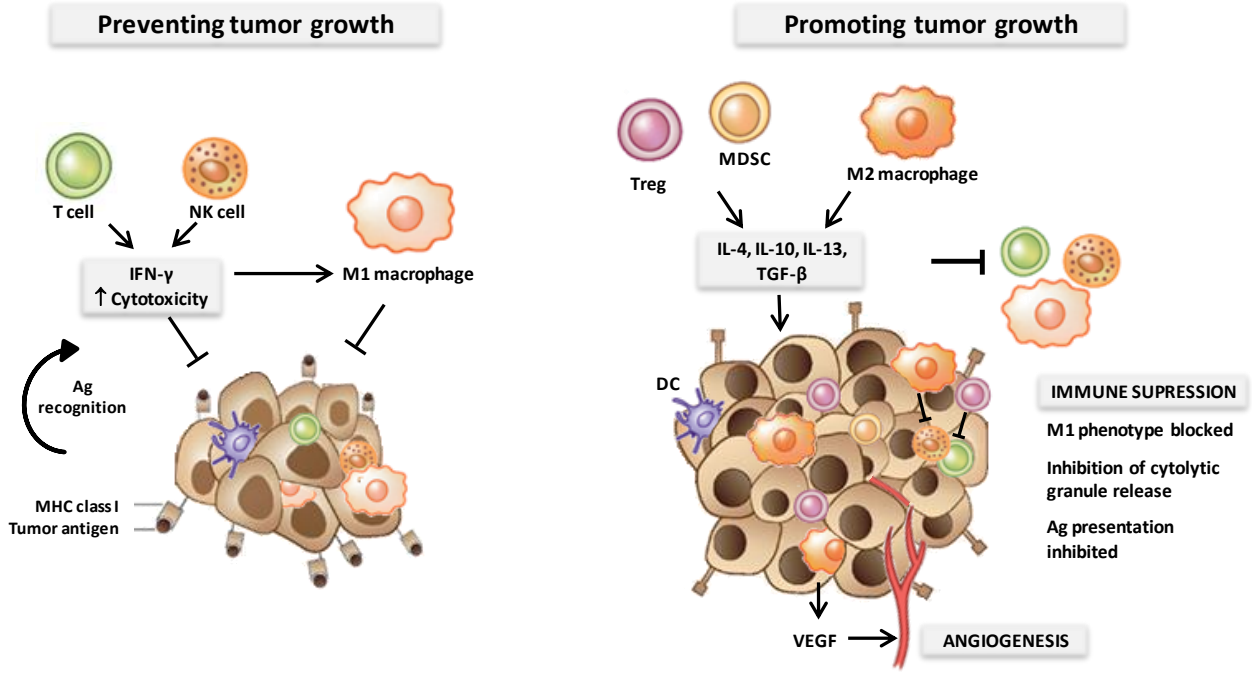

Figure 7. Schematic diagram illustrating some of the multiple cell types present in the tumor microenvironment that converge to support the tumorigenic niche. Tumor cells co-exist with a variety of cells that may either act to eliminate tumor cells or to promote tumor growth and progression. Immune cells recognize tumor cell-specific antigens (Ag) leading to their destruction and contributing to a growth-suppressive state. However, the tumor can act to eliminate the pressure by the immune system and some cells (e.g. tumor-associated macrophages) may later become educated by the tumor to acquire pro-tumorigenic functions, creating an immunosuppressive environment that supports tumor growth, angiogenesis and tumor invasion, by secreting pro-tumorigenic cytokines and growth factors (e.g., VEGF which supports angiogenesis). As the tumor grows, immune-suppressors such as myeloid-derived suppressor cells (MDSCs) and Treg cells are mobilized in response to specific cytokines (e.g., TGF-β), leading to disruption of antigen presentation by dendritic cells (DCs), inhibition of T and B cell proliferation/activation and NK cell cytotoxicity. M2-polarized macrophages and Tregs secrete anti-inflammatory T-helper 2 (Th2) cytokines (e.g., IL-4, IL-10, IL-13 and TGF-β), which suppress the anticancer immunity mediated by natural killer (NK) cells, cytotoxic $CD8^{+}$T cells and pro-inflammatory cytokines (e.g., IFN-γ). (Adapted from Quail et al. *Nat Med* 2013 [238] and Seruga et al. *Nat Rev Cancer* 2008 [248])

Table 3. Most commonly described mechanisms associated with immune evasion by tumor cells.

Interference with signal recognition and induction of anti-tumor immune response
✓ Loss of expression of surface antigens by tumor cells
✓ Downregulation of surface expression of HLA class I molecules on tumor cells
✓ Absence of costimulatory molecules or HLA class II molecules on tumor cells
✓ Suppression of immune responses by tumor cell products (e.g. TGF-β)
✓ Death receptor/ligand signaling and 'tumor counterattack'
✓ Low expression of costimulators on APC and inadequate cross-presentation of tumor antigens to T cells
✓ Alterations in TCR signaling in tumor-infiltrating lymphocytes (TIL)
Inadequate effector cell function in the tumor microenvironment
✓ Suppression of immune cells (e.g. T cells) by Tregs or myeloid-derived suppressor cells (MDSC)
✓ Apoptosis of effector T cells in the tumor
Development of immunoresistance by the tumor
✓ Lack of susceptibility to immune effector cells
✓ Immunoselection of resistant variants

3.1. The CNS microenvironment in brain tumors

The CNS provides unique microenvironmental conditions, which differ significantly from most other organs and tissues [249]. This is related to a certain extent to an active blood brain barrier (BBB), which confers to the CNS a selectively permeability around most blood vessels; such selective permeability, limits diffusion of molecules from the blood to the tissue, limiting the exposure of the brain parenchyma to circulating antigens and metabolites. In this regard, the BBB comprises tight junctions between endothelial cells surrounding the vessel and astrocyte foot processes [250]. The pericytes, a population of cells resident in the perivascular space, share a common basement membrane with the capillaries and provide mechanical stability to the endothelial-based capillaries [251]. Another unique feature of the CNS is its unique cellular composition, which includes several cells with potent immunoregulatory properties, and the lack of a (normal) lymphatic drain system [252]. Taken together, these factors contribute to explain the 'immune privilege' of the brain, which is often described as a tissue with diminished or absent immune responses [253]. However, this concept is more complex as this status is not uniform throughout the brain. Some brain regions are not protected from the (systemic) immune system in the same way as the brain parenchyma [250]. In addition, the resident myeloid cell populations are distinct in different regions of the brain,

since the ventricles containing the choroid plexus and the cerebrospinal fluid (CSF), the meninges, and the perivascular space have distinct immunological properties [253]. Despite all the above, at present it is known that circulating (systemic) immune cells are capable of migrating from cerebral vessels into both the perivascular space and the brain parenchyma in response to various stimuli and signals [254]. Migration of leukocytes is believed to occur in post-capillary venules, where the BBB is less strictly selective, with lower density of tight junctions and a perivascular space surrounding the vessels that does not exist in the brain capillaries (Figure 8) [254]. The mechanisms by which circulating leukocytes can cross the endothelial cell layer remain largely unknown; however, lymphocytes and leukemic cells, appear to migrate transcellularly across the endothelial cell layer using e.g. the VLA-4/VCAM-1 ligand-receptor complex to adhere to endothelial cells in the brain vasculature [255]. Following attachment of circulating immune system cells onto the vessel wall, they are subsequently often activated by chemokines [255].

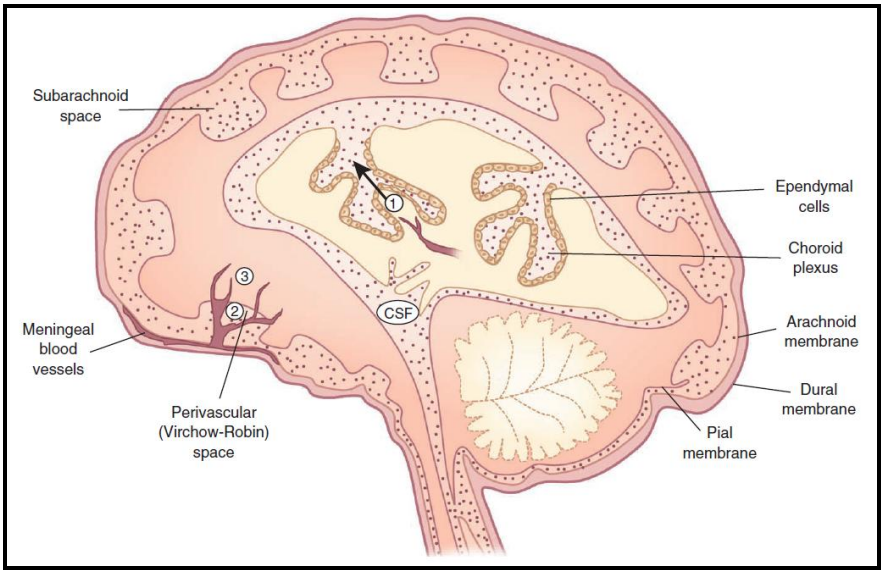

Figure 8. Schematic representation of the brain and the potential sites of immune cell entry into the CNS. T cells and monocytes may gain access to the brain through the choroid plexus by crossing through the ependymal layer, into the CSF (1), through the perivascular or Virchow-Robin space where the meningeal blood vessels branch into the subarachnoid space (2) and directly into the CNS parenchyma through postcapillary venules (3). (Reproduced from Ousman et al. *Nat Neurosci* 2012 [254])

3.1.1. CNS resident immune cells

Different subsets of myeloid cells are present in the healthy CNS, including the brain. Thus, parenchymal microglial cells are considered to be CNS resident macrophages. Myeloid cells which populate other brain compartments are generally referred to as macrophages, prefixed with their localization, e.g. choroid plexus, meningeal or perivascular macrophages

[249, 253]. Phenotypically, the distinction between parenchymal microglia from other brain macrophages has been based on the levels of expression of CD45 (leukocyte common antigen): microglial cells are characterized by low CD45 expression, whereas other macrophages are $CD45^{high}$ [253, 256]; in addition, human parenchymal microglial cells have further been reported to express $CD11b^{+}$ [257]. However, in many studies the term microglia, macrophages or microglia/macrophages is used to describe potentially mixed cell populations. Perivascular macrophages participate in antigen-presentation at the BBB, they have a high turnover rate and are constitutively replenished by circulating monocytes. In contrast, parenchymal microglia are differentiated tissue macrophages which are supposed to take up residency in the brain during embryonic development [258].

The spectrum of functional activities of these cells in the brain is as wide as for conventional macrophages, including phagocytosis, antigen presentation and migration. Some of the chemokines that have been related to migration of microglial cells/macrophages in the brain include: CCL21 via interaction with its CXCR3 receptor, CX3CL1 and CX3CR1, SDF-1α (CXCL12) via CXCR4, and the monocyte chemotactic protein 1 (MCP-1, CCL2) via CCR2 [253, 254, 259-261]. Among other phagocytic cell receptors, these cells might express the toll like receptors (TLRs) 1-9, immunoglobulin Fc receptors, scavenger receptors and complement receptors, phagocytosis being a major function of activated microglia/macrophages [253, 261]. In contrast, the ability of these cells for antigen presentation to T cells is more controversial. A pre-requisite of antigen presenting cells (APCs) is expression of Major Histocompatibility Complex (MHC or HLA) class II (for CD4 T cells) and MHC/HLA class I (for CD8 T cells) molecules together with costimulatory molecules. Microglial cells are usually considered the primary immune effector cells in the CNS, which are capable of generating significant immune responses. It has been suggested that upon stimulation, resident microglial cells can be rapidly activated via at least two functionally distinct morphological states, termed activated (which express only MHC-I) and reactive/amoeboid microglia (which express MHC-I and MHC-II in association with increased antigen presenting ability) [249, 261, 262]. However, several reports have identified those cells residing in the perivascular space or the meninges as the cells displaying the greatest ability to present antigens to infiltrating T cells for their stimulation [253, 263]. As macrophages infiltrate the perivascular space, infiltrated T lymphocytes recognize the antigens presented by these APCs and they will subsequently act as effector adaptative immune cells [263]. Indirect evidences also indicate that dendritic cells (DCs) are another group of candidate APC to migrate from the brain to lymph nodes [263, 264]; their CCR7-mediated chemotaxis facilitates lymph node entry through the high endothelial venules, being promoted by a CCL21 chemokine gradient [253].

3.1.2. Cellular composition of the cerebrospinal fluid

In recent years, several reports have provided detailed information about the composition of cerebrospinal fluid (CSF) as regards its cellular components. Overall, CSF is a paucicellular sample which mainly contains leucocytes typically at counts below 5 cells/µL. Around two-thirds of the whole white blood cell populations correspond to T cells (mainly $CD4^+$ and to a less extent also $CD8^+$ T-lymphocytes) and around 25% are monocytes. In contrast, B-lymphocytes, NK-cells, dendritic cells as well as neutrophils are only detected in some CSF samples from normal individuals at typically lower numbers [265, 266].

3.1.3. Immune cell infiltration in brain tumors

Several distinct subtypes of immune cells have been reported to infiltrate brain tumors, where they have been associated with a wide spectrum of functions [249, 251, 252]. From the different subtypes of brain tumors, glioblastoma multiforme (GBM) is among the most investigated subtype reported in the literature, due to its relatively high incidence and aggressive clinical behavior. Overall, these studies have shown that despite the presence of immune cells in GBM, the overall tumor environment is highly immunosuppressive, such environmental behavior depending on the type of immune cells present in the tumor tissue. In this section we will briefly review the main populations of immune cells that have been reported to infiltrate brain tumor tissues and their contribution to the behavior of the tumor niche, with special emphasis on meningiomas.

3.1.3.1. Myeloid cells

Myeloid cell populations that have been shown to infiltrate brain tumors include microglial cells and tumor-associated macrophages (TAM); in addition, myeloid-derived suppressor cells (MDSC) and DCs have also been identified among immune cell infiltrates in CNS tumors. Of note, all such myeloid cell populations partially overlap with native CNS tissue myeloid cells, which may make it difficult to determine whether in many tumors they correspond to tissue resident or newly-recruited cells.

Microglial cells & tumor-associated macrophages (TAM). Several studies have recurrently reported infiltration by microglial cells and TAM in both primary - e.g. gliomas [257, 267-271] and meningiomas [270, 272-277] -, and metastatic brain tumors [250, 270, 273]. Although a clear discrimination between both subtypes of myeloid cells may still require full definition, several studies have proposed the existence of highly discriminately phenotypes based on

marker combinations such as CD45/CD11b expression [257, 278, 279]. For example, Parney et al. [257] identified a median of 1.65% of $CD45^{low}CD11b^{+}$ cells (microglia) and 6.25% $CD45^{bright}CD11b^{+}$ (monocytes/macrophages) as inflammatory cells infiltrating human gliomas. At present, it is well known that tumor cells can secrete several factors which may potentially be responsible for the recruitment of microglia/macrophages; among others, these include MCP-1 (CCL2) and MCP-3 (CCL7), colony stimulating factor (CSF)-1, -2 and -3, SDF-1α and hepatocyte growth factor (HGF) [259, 280-285]. Whether myeloid cells infiltrating brain tumor tissues are a cause or a consequence of tumor progression, still remains controversial. Nevertheless, it is tempting to model brain myeloid cells on current concepts of macrophage plasticity, in which classically activated macrophages (M1) may promote anti-tumoral responses, whereas alternatively activated (M2) macrophages are predicted to be pro-tumoral. Polarization of TAM towards an M1 or M2 phenotype depends on the cytokine milieu and the local microenvironment.

Classically activated M1 macrophages are induced by IFN-γ and/or TLR ligation (through, e.g. microbial stimuli/LPS), as well as by cytokines - e.g. tumor necrosis factor (TNF)-α and GM-CSF -. Polarization towards M1 cells is typically observed in the presence of high interleukin (IL)-12 and IL-23 production, but low IL-10 levels; M1 macrophages participate as inducer and effector cells in polarized Th1 responses, through production of pro-inflammatory cytokines, including IL-1, TNFα and IL-6. In turn, these cells up-regulate nitric oxide synthase 2 (NOS2) expression therefore producing reactive oxygen and nitrogen species. M1 macrophages have antigen presentation ability, and they mediate innate immune responses against intracellular parasites and tumors [246, 247, 286].

The designation M2 covers several forms of macrophage activation which differ from the classical M1 cells, including cells exposed to IL-4, IL-13 and glucocorticoid/corticosteroid hormones. M2 macrophages share an $IL\text{-}12^{low}$, $IL\text{-}23^{low}$, $IL\text{-}10^{high}$ phenotype in association with variable production of anti-inflammatory cytokines, depending on the signals leading to their activation. In addition, M2 cells have high amounts of scavenger, mannose, and galactose-type receptors (e.g. CD163, CD204 and CD206) and they show up-regulation of arginase 1 (Arg1), and a shift of the arginine metabolism towards production of ornithine and polyamines. Furthermore, differential regulation of the production of distinct components of the IL-1 system takes place in polarized macrophages, M2 cells being associated with low IL-1αβ, high IL-1 receptor antagonist, and high decoy type II receptor levels. In general, M2 cells participate in polarized Th2 immune responses, they are present in established tumors where they promote tumor progression, tissue repair, and remodeling; they are typically associated with lack of cytotoxic activity, through the blockade of $CD8^{+}$ T-cell infiltration and proliferation, at

the same time they display immunoregulatory functions [246, 247, 286]. Another major pro-tumoral role of M2 macrophages is related to their effects on promoting angiogenesis through the release of pro-angiogenic growth factors such as VEGFA, a process which is essential for tumor progression.

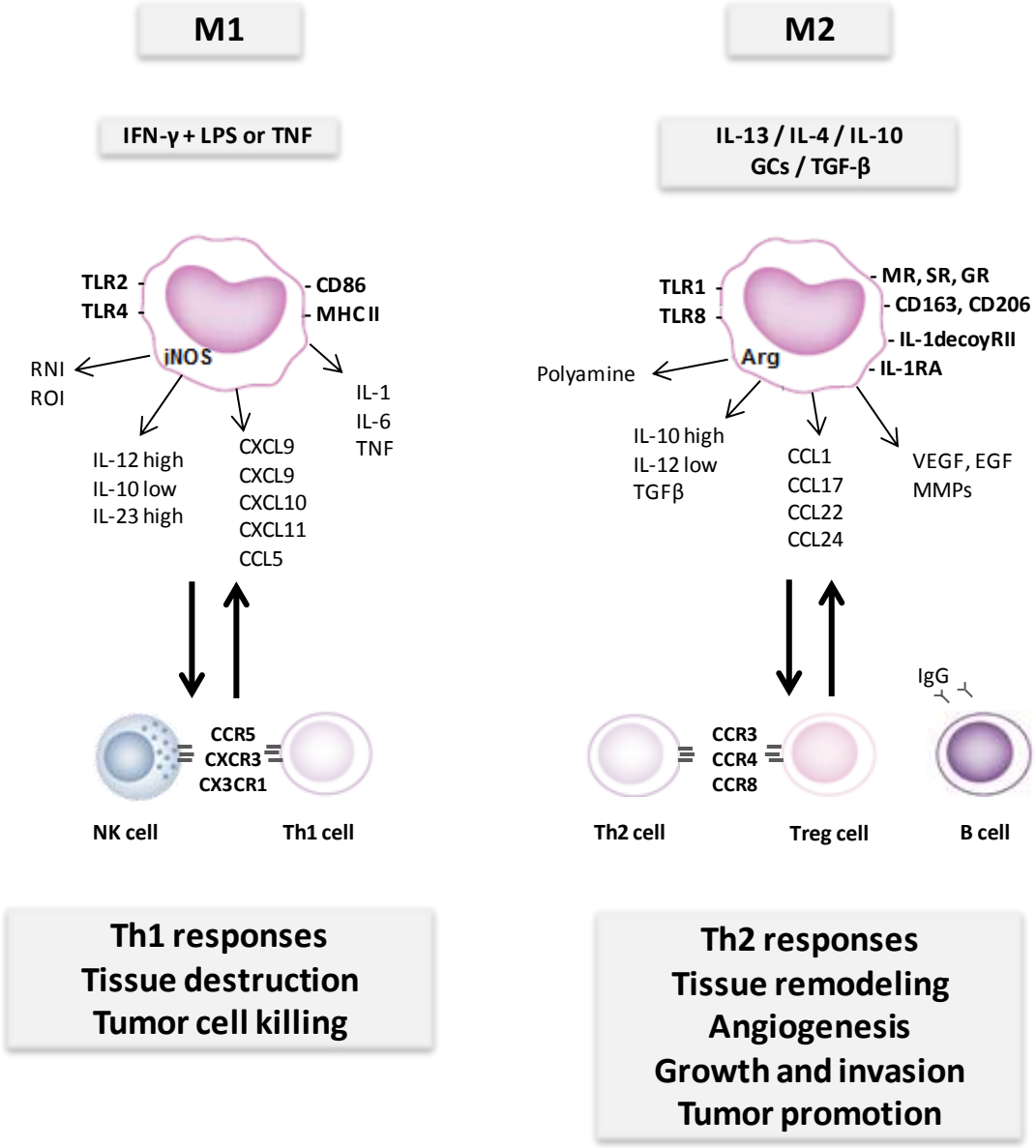

Figure 9. The two major macrophage (polarized) phenotypes in tumor tissues and their interaction with infiltrating lymphoid cells. Tumor-associated macrophages can have either beneficial anti-tumoral or pro-tumoral effects in cancer, depending on the cellular and tissue microenvironment. The environment-derived signals and selected functional properties of two main polarized macrophage populations, as well as the different cytokines, chemokines, and receptors expressed, are shown. **Abbreviations:** IFN-γ, interferon-γ; LPS, lipopolysaccharide; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; TLR, Toll-like receptor; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; IL, interleukin; CCL, chemokine (C-C motif) ligand; NK, natural killer; TGF-β, transforming growth factor-β; SR, scavenger receptor; MR, mannose receptor; GR, glucocorticoid receptor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; MMPs, matrix metalloproteinases. (Adapted from Mantovani et al. *Trends Immunol* 2004 [290] and Biswas et al. *Nat Immunol* 2010 [286])

Although macrophage polarization towards an M1 vs M2 phenotype depends on the cytokine and extracellular signals that predominate in the tumor microenvironment, several transcription factors and signaling pathways are required to be activated at the intracellular level, including the nuclear factor κB (NF-κB), STAT3 and HIF-1-mediated activation pathways [287]. In this regard, whereas some cytokines (e.g. IL-1 and TNFα) in the tumor milieu can activate the NF-κB pathway, others such as IL-10, are important activators of the oncogenic STAT3 signaling pathway which favors tumor survival and drives abortive activation of immune cells [287].

Despite all the above, and the potential utility of the M1/M2 classification, it should be noted that such M1/M2 balance somewhat represents an oversimplification of TAM functional profiles, as it does not fully reflect the complexity of macrophage activation, which is often tuned differently in response to different tissue microenvironments [238]. At present, it is not entirely clear how macrophages switch phenotypes. Previous studies have suggested that hypoxia might be the major factor in mediating the transition from tumor suppressing to tumor promoting macrophages [238]. Reversion of an M2 back to an M1 phenotype has also been reported. For example, disruption of NF-kB signaling in an ovarian cancer model resulted in an M2-to-M1 switch, the recruitment of NK cells and subsequent tumor regression [288]; similarly, macrophage depolarization from an M2 phenotype by inhibition of CSF-1R was associated with robust regression of already established high-grade gliomas [289]. Altogether these studies highlight a potential therapeutic opportunity in which re-education of TAM might have beneficial anti-tumorigenic effects on the outcome of the disease.

As explained above, most studies available on the microglial cells and macrophages of brain malignancies have focused on gliomas/GBM and the mechanisms which are responsible for their altered microenvironment. Some of these studies demonstrated that microglial cells and brain macrophages have the potential to exert anti-tumoral effects *in vitro* [291-293]. In this regard, Galarneau et al. [293] reported that macrophage depletion results in an increased volume of glioma; these authors further found the immune infiltrate of gliomas to reflect type 1 responses, with $CD11b^+$ cells being the main source of TNFα, in the presence also of high levels of MCP-1 and IL-1β, but low levels of IL-4 and IL-10. Overall, these results suggest that the brain is either equipped, or it can recruit, cells with potential to act against brain tumors. However, these functions may be overwhelmed by pro-tumoral elements. Indeed, most studies in microglia and brain macrophages have shown a pro-tumoral phenotype, associated to an immunosuppressive microenvironment and promotion of tumor growth and invasion. Therefore, microglia/macrophage infiltrates in gliomas have been mainly associated with an M2 polarization, TAM expression of M2-associated markers such as CD163, CD204 and CD206,

as well as IL-10 and arginase 1 production [294-297]. In line with this, Hussain et al. [271, 298] investigated the functional profile of myeloid cells isolated from patients with malignant glioma, their results showing that, despite those cells expressed significant levels of TLRs, they did not secrete pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNFα) and they lacked expression of co-stimulatory molecules (e.g. CD86, CD80, and CD40) which are critical for subsequent T-cell activation; furthermore, they showed that STAT3 signaling might also be involved, as STAT3 inhibition was accompanied by an enhancement of immune responses and up-regulation of several key intracellular signaling molecules that regulate T-cell activation [299]. Consequently, these results indicate that blockade of microglia/macrophage infiltration and/or their pro-invasive effects could represent a potentially beneficial therapeutic strategy in malignant gliomas. However, it should be emphasized that in general, the immunosuppressive microenvironment of gliomas results from a bidirectional communication, in which the microglia/macrophage cell compartments secrete multiple cytokines and growth factors that can directly or indirectly lead to tumor proliferation (e.g. EGF) and invasion (e.g. metalloproteinases), as well as angiogenesis (e.g. VEGF) [262]; at the same time glioma cells secrete immunosuppressive factors such as TGF-β and IL-10, that induce and promote an impaired functionality of microglia/macrophages which limit their ability to cooperate with T-cells to generate effective anti-tumoral responses [262, 300].

Of note, despite all the insights brought by the above studies into the microenvironment of gliomas, to the best of our knowledge, infiltration by macrophages has also been reported in meningiomas [270, 272-277], but no studies have investigated the effect of such cells in the immune context of meningiomas, as well as its possible polarization and functional profile.

Myeloid derived suppressor cells (MDSC). One of the most prevalent mechanisms of immune evasion in cancer patients is through the immunosuppressive activity of MDSCs. MDSCs are an heterogeneous group of immature myeloid cells characterized in humans as being $CD11b^{+}CD14^{+}CD15^{+}HLA\text{-}DR^{-}CD33^{+}$ cells [301]. MDSCs are mobilized during tumorigenesis and infiltrate developing tumors, where they promote tumor vascularization and disrupt major mechanisms of immunosurveillance, including antigen presentation by DCs, T cell activation, M1 macrophage polarization and NK cell cytotoxicity [302]. Presence of MDSCs in the immune infiltrates of human brain tumors has not yet been described. However, characterization of MDSCs infiltrating the GL261 glioma mouse model has been discussed in some detail [295]; in this mouse glioma model, MDSCs show a substantial overlap with tumor associated macrophages, they share phenotypic features of both M1 and M2 polarized macrophages, and

they display a considerable plasticity in their function and phenotype, depending on the surrounding microenvironment. Furthermore, circulating $CD33^{+}HLA\text{-}DR^{-}$ MDSC have been detected in the peripheral blood of GBM patients at greater levels than in healthy donors [303], and healthy donor-derived human $CD14^{+}$ monocytes may acquire MDSC-like properties when exposed to glioma cells, these including an increased production of immunosuppressive IL-10, TGF-β and B7-H1 and an increased ability to induce apoptosis of activated lymphocytes [304].

Dendritic cells (DCs). Another subtype of myeloid cells that may be specifically recruited to brain tumors are the DCs. Although the brain does not has a standard lymphatic system like other tissues in the body, the perivascular space has been claimed to potentially act as a route for lymph to drain into the cervical lymph nodes, and thus, act as a flow channel for the adaptive immune system [264]. In the context of glioma, DCs have been most thoroughly investigated in the GL261 mouse glioma model. In this animal model, infiltrating ($CD11c^{+}$) DCs have been shown to display little or no expression of costimulatory molecules (CD40, B7.1, B7.2), they are unable to stimulate T cells but instead, they promote development of Tregs [305]. Another study based on a transgenic mouse model of spontaneous astrocytoma also reported $CD11c^{+}CD11b^{+}CD8^{-}$ DCs infiltrating the tumor, which were potentially inefficient for antigen cross-presentation and priming of CD8 T-cells [306]. In humans, analysis of circulating myeloid and plasmacytoid DCs in the peripheral blood of patients with glioma has shown decreased numbers *vs.* healthy controls [307]. Tyrinova et al. [308] investigated monocyte-derived DCs from brain glioma patients generated *in vitro* in the presence of IFNα and GM-CSF, and they found impairment of DCs to be potentially involved in the pathogenesis of the tumor. To the best of our knowledge, no study has investigated so far the presence, distribution and/or functionality of DCs in meningiomas.

3.1.3.2. Lymphoid cells

Tumor-infiltrating lymphocytes (TIL) are also a systematic component of the microenvironment of brain tumors [253], suggesting that these cells may be critically involved in tumor growth, progression and/or control. Of note, myeloid cells engage in complex bidirectional interactions with the lymphoid cells in order to exert their function in the tumor microenvironment [286]. TIL usually consist of T-cells and, to a less extent, also NK-cells and B lymphocytes [237, 239].

T-cells. T lymphocytes ($CD3^+$) fall into two major broad categories: $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs). Both have been described to infiltrate brain malignancies, such as meningiomas [272, 309-311], gliomas [312-315], and metastatic lesions from other tumors [314, 315]. Several studies have further analyzed the relationship between T cell infiltration and patient outcome, with controversial results [316]. One potential explanation for the controversial results may be the distinct role played by the different T-cell subsets infiltrating the tumor. Usually, high levels of $CD8^+$ CTLs are related to greater anti-tumoral activity, whereas high levels of CD4+ Th cells, particularly some subsets of Th cells, are viewed as being associated with a role favoring tumor development [250]. In line with this, Yu et al. [313] reported a high $CD8^+/CD4^+$ T-cell ratio in primary brain tumors to be associated with less aggressive disease, and others have associated greater levels of infiltration by $CD8^+$ T-cells with a better survival [317-320].

$CD4^+$ Th cells (Th1 and Th2) deserve a specific comment since these cells seem to play a very important role in regulating the phenotype of TAM. In this regard, Th1 cells can drive classical M1 polarization of macrophages through production of IFN-γ, while Th2 cell-derived IL-4 and IL-13 direct M2 polarization of macrophages. In turn, IL-4-activated macrophages express chemokines such as CCL17, CCL22 and CCL24, whose specific receptors (CCR4 and CCR3) are expressed by Th2 cells [286]. Of note, analysis of the activation profile of TIL in malignant glioma has shown predominance of type 2 immune responses in the intratumoral microenvironment, in association with expression of Th2 type cytokines (e.g. IL-4, IL-10 and IL-6); these findings might contribute to explain the 'immunosuppressive microenvironmental status' of these tumors [321-324]. In order to investigate the Th1/Th2 balance in different types of brain tumors, Kumar et al. [325] analyzed IL-12 (related to Th1 responses) and IL-10 (related to Th2 responses) serum levels in patients with meningioma, anaplastic astrocytoma and GBM; overall, these authors found a significant reduction in serum IL-12 and increase in serum IL-10 in patients *vs.* controls. However, it should be noted that such balance was much closer to the normal values among meningioma (IL-10 levels among meningioma patients were similar to those of the controls), than glioma patients, suggesting a less predominant type 2 immune response in the former patient group. In line with this observations, Shimato et al. [326] have recently reported on the *in vitro* production of IFN-γ (Th1) and IL-5 (Th2) by freshly-isolated, *in vitro* stimulated, peripheral blood mononuclear cells (PBMCs) from patients with GBM and meningioma; overall, both patient groups showed a modest decrease in the amount of secreted IFN-γ when compared to healthy subjects, while a significant elevation of IL-5 levels was found only for recurrent GBM patients. Consequently, when the IFN-γ/IL-5 cytokine ratio was considered, no predominant Th1 or Th2 bias was found among meningioma patients,

while patients with both primary and recurrent GBM exhibited a significantly decreased IFN-γ/IL-5 ratio in favor of a predominance of Th2 immune responses.

In recent years, several reports have specifically investigated the presence of $CD4^{+}CD25^{high}FOXP3^{+}CD127^{low}$ regulatory T cells (Tregs) in brain tumors, since these cells have been shown to play an important role in the control of immune responses via the suppression of proliferation of other T cells in the microenvironment, through either direct cell-to-cell contact-dependent mechanisms or indirectly by IL-10 and TGF-β secretion [250]. Thus, Tregs have been shown to infiltrate both primary and metastatic brain tumors [327]. Of note, Tregs infiltrating brain tumors have been shown to be fully activated and to strongly suppress proliferation and cytokine production by TIL, thereby contributing to a more aggressive clinical behavior of high-grade brain tumors [327]. In this regard, a strong correlation has been reported in GBM between immunosuppression and presence of Tregs in the tumor microenvironment [328, 329], and tumor infiltration by Tregs has also been shown to correlate with tumor grade [330]; in addition, depletion of Tregs in animal models has been associated with prolonged survival and infiltration by non-immunosuppressive myeloid cells [331-334]. In the tumor microenvironment, production of specific chemokines (e.g. CCL22) and cytokines (e.g. TGF-β) appears to be associated with preferential recruitment of Tregs and promotion of tumorigenesis [246, 286]. Moreover, tumor infiltrating Tregs can also affect the function of TAM favoring polarization towards an M2 suppressive phenotype in the tumor microenvironment [286].

Natural killer (NK) cells. NK cells consist of cytotoxic effector lymphocytes that play an important role in anti-tumoral innate immune responses through e.g. apoptotic killing of tumor cells [250]. NK cells exert their effects via two major cytotoxic pathways. On one side, NK-cells are rich in perforin- and granzyme-containing granules, that once released, lead to the perforation of the cytoplasmic membrane of targeted cells and their subsequent death by apoptosis; on the other hand, they constitutively express the CD95-ligand and TNF on the cell surface which bind to apoptotic receptors on the target cell, also leading to apoptosis [335]. In addition, NK cells secrete a variety of cytokines and chemokines (e.g. IFN-γ), which exert immunomodulatory effects such as priming of Th1-biased T-cell responses and classical M1 polarization of macrophages [286, 335]. Thus, NK cell infiltration into tumors has been correlated with a more pronounced anti-tumor effect and a better patient outcome [239]. However, it should be noted that NK cells have been identified using CD57 or CD56, both phenotypic markers being characteristic but not specific of NK cells; in fact, NK cells should be better characterized as $CD3^{-}CD56^{+}$ and/or $CD57^{+}$ cells to exclude $CD56^{+}$ and $CD57^{+}$ T-cells

[336]. In brain tumors, the tumor-suppressing role of NK cells has been demonstrated both *in vitro* [337, 338] and *in vivo* [339-342]. Moreover, these cells have been identified in both primary (e.g. meningiomas and gliomas) and metastatic brain neoplasms [267, 315, 320, 343-346]. However, the level of tumor infiltration by NK cells tends to remain low and the functionality of such cells is often affected by factors released by the tumor and/or other immunosuppressive cells [336]. As an example, TGF-β secreted locally be tumor cells and other infiltrating cells down-regulates the expression of the NKG2D activating receptor on NK cells isolated from glioblastoma patients, at significantly more pronounced levels than NK cells from meningioma patients [347].

B cells. The specific role of B-lymphocytes in the development of brain tumors remains unclear. Some reports identified B cell infiltration in meningiomas [272, 309, 348, 349] and gliomas [346, 350, 351]. However, such B cells only represented a minor fraction of the immune cell infiltrates in these tumors. In other types of cancer, tumor-infiltrating B cells have been associated with the recognition of a wide variety of tumor antigens, and they have been claimed to closely interact with T cells and other immune cells, in association with a more favorable outcome [352]. In this regard, B cells can indeed act as APCs and, therefore, they may be relevant for inducing $CD4^+$ T cell-dependent $CD8^+$ memory T cells that help to control tumor invasion, spread and metastasis [352]. In a GBM model, Candolfi et al. [353] showed that B cells can act as APCs for T-cells and potentially play a critical role in T-cell-mediated antitumor immunity and T cell-dependent tumor regression within the CNS. Similarly, a recent report on meningioma-infiltrating B cells provided clear evidence for the presence of antigen-experienced B-lymphocytes [309]. However, presence of tumor infiltrating B cells may have a paradoxical effect, as some reports also found B cells to suppress the development of immune responses in some tumors [354], and to directly regulate macrophage effector functions through IL-10 production, which may activate an M2 macrophage phenotype and promote tumorigenesis [286].

3.2. Evaluation of the cellular composition of tumor tissues

Tumors typically harbor a heterogeneous and variable cellular composition. Until now, several different techniques have been used for the identification and characterization of the different cell populations coexisting in tumor tissues. Among such techniques,

immunohistochemistry (IHC) is the most widely used method in diagnostic surgical pathology of solid tumors [355]. This technique combines staining with antibodies for localization and identification of specific antibody-targeted antigens in a cell or tissue specimen by light microscopy [356]. Therefore, it allows the observer to distinguish between cancer cells and other different types of non-neoplastic cells, through combined assessment of cell morphology and detection/recognition of specific molecules in one or more subsets of cells. This method permits semi-quantitative evaluation of the cellular components of a tumor sample, and determination of the specific localization of a cell population in the tumor tissue. However, it has also some limitations, which relate to: the subjective nature of data interpretation with a relatively high degree of inter-observer variability; usually it does not allow simultaneous identification of all different cell populations in the sample, and; the identified cells cannot be isolated in sufficient numbers for their further complete (e.g. molecular) characterization, even when laser-microdissection techniques are used [357].

More recently, multiparameter flow cytometry (MFC) has also been used for the identification and characterization of heterogeneous cell populations coexisting in tumor samples [358]. However, whereas MFC is currently applied in routine clinical diagnosis and classification of hematological malignancies (e.g. leukemia and lymphoma) [359], its application to the study of solid tumor tissue samples, remains rather limited. This is mainly due to the fact that MFC cannot be directly applied to the study of solid tumors tissues, since it requires prior preparation of single cell suspensions from the tumor tissue specimen [360].

3.2.1. Principles of multiparameter flow cytometry (MFC)

The basic principle of flow cytometry relies on the interrogation of single particles (or cellular 'events') which are suspended within a fluid stream, as they pass aligned one by one in front of one or more light sources – e.g. lasers – at relatively high speed (e.g. $>10^3$ cells/second) [361]. As individual cells are hit by the laser, information is generated because of light scatter - forward light scatter (FSC) is a measure of cell size and sideward light scatter (SSC) is a measure of the intracellular granularity complexity - [358], and multiple fluorescence emissions due to the presence of naturally or artificially bound (e.g. via fluorochrome-conjugated antibody reagents) fluorochromes inside and/or outside the cell. The information generated about single cells is then stored in a digital format in a computer. The key advantage of flow cytometry is that a very large number of particles/cells can be evaluated in a very short time, information being generated for multiple parameters in a single cell basis, which confers MFC unique analytical capabilities *vs.* other technologies [358, 361].

The first rather common application of MFC to the study of solid tumors has relied on the quantification of the tumor cell DNA contents for both the identification of aneuploid cell populations [359] and the analysis of the cell cycle distribution of tumor cells [362]. As an example, several studies have long shown a relationship in meningiomas between both the tumor cell DNA contents and their proliferative index as analyzed by MFC, and the grade of malignancy of the tumor [48, 363-365].

In the last three decades, immunophenotyping has become a major application of MFC in oncology, due to the availability of a vast number of monoclonal antibodies conjugated with a wide variety of fluorochromes detectable at different wavelengths and that might be used to simultaneously identify membrane, cytoplasmic and/or intracellular proteins in single cells [359, 361]. Based on the specific immunophenotypic profiles obtained with different cocktails of antibodies, clear identification and discrimination among distinct cell populations coexisting in a sample can be easily achieved, based upon quantitative differences for one or more phenotypic variables measured on each cell in the sample [358]. Most interestingly, MFC sorting instruments also allow simultaneous physical separation of multiple (viable) cell populations coexisting in a sample with a high (>90%) purity, for their further analysis using other techniques (e.g. molecular and functional studies) [358, 361].

However, the major disadvantage of MFC for the study of solid tissues relies on the loss of all information about the tissue architecture and the spatial relationship between the different cells coexisting in a tumor sample [357]. Such type of analysis is also associated with the presence of increased amounts of cellular and tissue debris, due to the need to apply mechanical and/or enzymatically tissue disaggregation procedures for the preparation of single cell suspensions. Since sometimes debris are difficult to be excluded from the populations of 'cellular events', usage of a DNA-specific dye (e.g. DAPI or DRAQ5) to positively select for cells showing a high DNA content, is highly recommended [360, 362].

3.2.2. MFC immunophenotypic studies in meningiomas

Until now, several studies have used MFC immunophenotyping for the phenotypic characterization of neoplastic cells and the identification and/or characterization of the infiltrating cell populations in CNS tumors. Once again, most of these studies have focused on malignant gliomas and only a few have investigated meningiomas. In gliomas, MFC has been applied both to the analysis of tumor infiltrating macrophages/microglia [257, 271, 278, 296, 298, 306, 366-369], MDSCs [295] and lymphocyte subsets such as T-cells [306, 313, 321, 328, 330, 331, 369, 370], both in experimental and primary human tumor samples. In addition, MFC

immunophenotyping has also been used for the identification of subpopulations of tumor stem cells (e.g. $CD133^+$ tumor cells) and/or the evaluation of the pattern of expression of neural/glial lineage-specific differentiation markers (e.g. PDGFR-α, A2B5, O4, O1, CD44, CD56, NG2, GFAP) among different histopathological subtypes of gliomas [371-374].

In contrast, few studies have been reported in which MFC immunophenotyping has been used to characterize neoplastic and/or immune infiltrating cell populations in meningiomas samples, most of these studies being restricted to the analysis of a few individual phenotypic markers. Thus, Rooprai et al. [375] used MFC to evaluate CD44 expression in meningiomas. More recently, Rath et al. [376] reported on the immunophenotypic features of a purified population of tumor-initiating cells derived from an atypical meningioma, showing that such cells expressed CD44 and the activated leukocyte adhesion molecule (ALCAM) CD166 as evaluated by MFC. Regarding tumor-infiltrating immune cells, Asai et al. [274] identified 24% ± 3.7% of CD68-positive macrophages and/or microglial cells in meningiomas, which appeared to be heterogeneous, potentially reflecting various functional states and roles in the regulation of tumor growth. In turn, Yu et al. [313] investigated the TIL profile of meningiomas, showing infiltrates to consist of $CD3^+$ T-cells (median of 5.25%), with a low $CD4^+/CD8^+$ ratio (CD4/CD8 ratio of 0.25), indicating a higher infiltration by $CD8^+$ T-cells. In a more recent report, infiltration by $CD4^+FoxP3^+CD25^{high}CD127^{low}$ Tregs was evaluated in patients with several different subtypes of brain tumors; results showed no Treg accumulation in meningiomas, while GBM and metastic brain tumors showed massive infiltration by regulatory T-cells [327]. Similarly, Waziri et al. [370] evaluated the TIL of several brain lesions and they reported infiltration by $CD3^+$ cells and Tregs in meningiomas to be of 1.7% ± 0.7% and 0.6% ± 0.2%, respectively; these authors also found a unique cytokine production profile among the TIL from meningioma samples with high IFN-γ and low IL-4/IL-13 and IL-10 cytokine expression levels. To the best of our knowledge, no study has been reported so far in which the immunophenotypic features of meningioma tumor cells have been investigated by MFC.

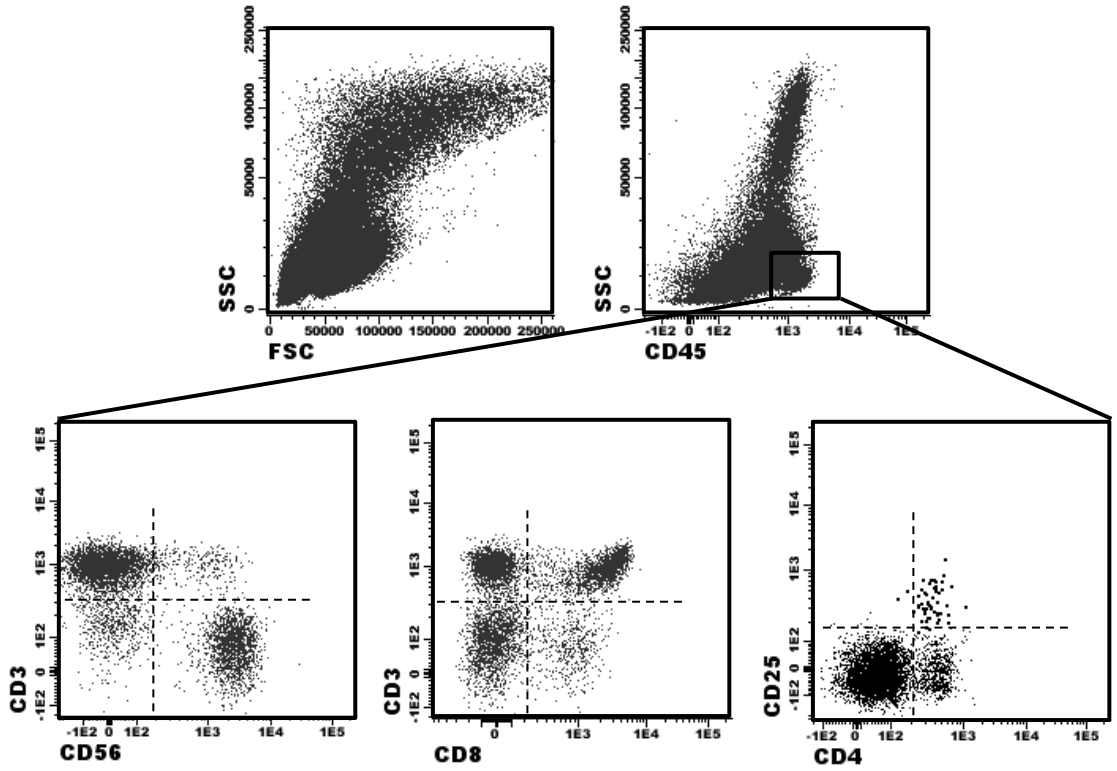

Figure 10. MFC immunophenotypic analysis of different subpopulations of tumor-infiltrating lymphocytes ($CD45^{hi}$) in meningiomas. In the upper panels, the whole tumor is represented in a forward light scatter (FSC) *vs.* sideward light scatter (SSC) dot-plot and CD45 *vs.* SSC dot-plot. In the lower panels, a dot-plot representation of the CD56 (x-axis) *vs.* CD3 (y-axis) markers for NK-cells and T-cells, respectively, and the CD8 (x-axis) *vs.* CD3 (y-axis) markers for cytotoxic ($CD3^{+}CD8^{+}$) and helper ($CD3^{+}CD8^{-}$) T cell subsets, are shown; the $CD4^{+}$ T cell subset are further subdivided into $CD4^{+}CD25^{hi}$ Treg cells.

Chapter II – Hypothesis and Objectives

Despite meningiomas are generally considered benign and frequently curable tumors, in practice, a significant proportion (≈20%) of these tumors will eventually relapse with an adverse impact on overall patient survival. For decades, it is known that such heterogeneous clinical behavior is directly related in part, to the wide range of histopathological patterns and biological features of this group of CNS tumors. In fact, the WHO grade of meningiomas based on the morphological and histopathological features of the tumor, together with the degree of tumor resection, are the most relevant factors for prognostic stratification of meningiomas. However, the great majority of meningiomas fall into the WHO grade I/benign histopathological category and, in absolute numbers, most recurrences occur among this subgroup; at the same time, still some grade II/III meningiomas are long term recurrence-free survivors. Altogether, these findings point out the need for additional parameters that would contribute to a better stratification of those tumors included in individual WHO grades. In this regard, an increasingly high amount of data about the genetic and molecular alterations of these tumors has been generated in the last decades, allowing for the identification of multiple recurrently altered chromosomal regions and candidate target genes, tumor cytogenetics also emerging as a major source of biological variability in meningiomas. In addition, once combined with histopathology, tumor cytogenetics has been claimed to contribute to an improved prognostic stratification of meningiomas (particularly of grade I tumors) and thereby also, the management of individual patients. Of note, the presence of multiple (≥2) chromosomal alterations has been recurrently associated with a poorer outcome among grade II/III meningiomas, as well as within WHO grade I/benign tumors.

In recent years, a few classifications have been proposed for prognostic stratification of meningioma patients based on combined assessment of tumor histopathology and cytogenetics, but ultimately, they have not been adopted in routine clinical practice. Among other reasons, this is most probably due to the fact that these new classifications fail in identifying both a good prognosis group of patients who are cured, in contrast to a poorer prognosis subgroup with a very high (close to 100%) recurrence risk. Therefore, additional criteria and/or prognostic scoring systems are also needed for better risk stratification of meningiomas already at diagnosis, opening the way to improve the clinical decision-making process as regards both the therapy administered after surgery and patient follow-up strategies.

Currently, it is well established that the genetic/genomic profile of tumor cells is closely associated with the behavior of the tumor from both a biological and a clinical point of view. However, increased number of evidences indicates that the tumor microenvironment

might also play a critical role in tumor growth and aggressiveness. In this regard, infiltration by immune cells in response to signals released by the tumor itself have emerged as particularly relevant components of the tumor microenvironment in contributing to clinical outcome and response to treatment in many different types of tumors. This is due to the fact that the different types of tumor-infiltrating immune cells may play a wide spectrum of roles and functions within the malignant lesion, with the potential to contribute either to eliminate or to promote malignancy. Thus, comprehensive analysis of the type of cells infiltrating the tumor, as well as of the level of infiltration by such cells, and their potential interactions with neoplastic cells, may also represent critical elements in the understanding of the biology and clinical behavior of meningiomas, in addition to tumor cytogenetics and grade. In this regard, it should be emphasized that specific genetic alterations in the tumor cells may also affect the type of immune cells recruited to the tumor environment and/or the role that these latter cells will play in the behavior of the tumor.

Despite all the above, and in contrast with the high number of reports about the genetic and molecular alterations of meningiomas, current knowledge about the different non-neoplastic cell populations infiltrating meningiomas and the protein - expression - profiles of the tumor cells, remains very limited, being usually restricted to the immunohistochemical analysis of a limited panel of cell-lineage and proliferation-associated markers. Even more, despite multiparameter flow cytometry is a well-suited method for the assessment of protein expression profiles of single cells, for simultaneous identification and characterization of the different cell populations coexisting in a sample, few reports have focused on the MFC immunophenotypic identification and characterization of meningioma tumor cells, including neoplastic and other infiltrating cell populations within the tumor. In addition, no study has been reported so far in which the potential association between the immunophenotypic profile of such cells and the clinico-biological features of the disease had been investigated.

Based on the above background, the ***general objective*** of this study was to simultaneously investigate the cytogenetic and the protein expression profile of meningioma cells and other cells infiltrating the tumor, and the potential association of these features with other clinical and biological characteristics of the disease. Our ultimate goal was to better understand the biology of meningiomas at both the tumor cell and the tumor microenvironment levels, and to establish the potential utility of the newly generated information for prognostic stratification of meningiomas. For this purpose, the following four ***specific objectives*** were pursued:

1. To analyze the overall cellular composition and the immunophenotypic profiles of the different (major) populations of tumor and infiltrating immune cells in meningiomas, using MFC immunophenotyping;

2. To investigate the potential association between the number and immunophenotype of the immune cells infiltrating meningiomas, and the clinical and biological features of the disease, including tumor cytogenetics and gene expression profiles;

3. To determine the potential relationship between the immunophenotypic profile of meningioma neoplastic cells and both the cytogenetic and gene expression profiles of such tumor cells, as well as the clinico-biological behavior and the outcome of the disease;

4. To construct a prognostic scoring system for risk stratification of meningioma patients based on the most relevant clinical and biological features of the disease, including tumor grade and tumor cytogenetics.

Chapter III – Material and Methods

1. Patients and samples

Patients included in this doctoral thesis were diagnosed with meningioma at the Neurosurgery Service of the University Hospital of Salamanca (Salamanca, Spain). Prior to entering the study, each patient gave his written informed consent to participate according to the Declaration of Helsinki protocol, and the guidelines of the local Ethics Committee. For each case, information about the histopathological characteristics of the tumor for their further classification according to the WHO criteria [6], as well as about the most relevant clinical and biological features of the disease (e.g. age, gender, tumor localization, brain edema, treatment, recurrence-free survival and overall survival), was collected. According to the extent of brain edema, patients were classified as having light (smaller or equal to the volume of the tumor), moderate (doubling the volume of the tumor) and severe edema (more than twice the volume of the tumor). In addition, each sample was characterized according to the interphase fluorescence in situ hybridization (iFISH) cytogenetic profile of the tumor cells as diploid, carrying an isolated monosomy 22/del(22q) and as having complex karyotypes, the latter being defined by the presence of cytogenetic alterations (losses and/or gains) involving ≥2 chromosomes [73].

For each tumor sample, representative parts of diagnostic left-over fresh tumor tissue specimen(s) were frozen in liquid nitrogen immediately after surgical removal, and stored at -150ºC (freshly-frozen samples) until used for further analyses. In addition to the tumor tissue specimen, ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood (PB) samples were also collected at diagnosis from each patient and processed in parallel.

1.1. Patients and samples used for multiparameter flow cytometry (MFC) immunophenotyping and gene expression profiling (GEP) studies

Overall, 75 meningioma patients (20 males and 55 females; mean age of 60 ± 14 years; range: 23 to 84 years), and 78 tumor samples, were included in this part of the study. According to their localization, tumors were distributed as follows: parasagittal, 9 cases (12%); convexity, 21 (27%); parasagittal and convexity, 8 (10%); tentorial, 2 (3%); cranial base, 25 (32%); spinal tumors, 12 (15%), and; one case (1%) corresponded to an intraosseous tumor. Brain edema was found in 37/75 (49%) patients. According to its extent, it was evaluated as light in 11 cases (15%), moderate in 16 (21%) and severe in 10 cases (13%); the remaining 38

(49%) cases showed no edema. According to the WHO criteria, 64 (82%) of these tumors were benign/grade I meningiomas, 11 (14%) were grade II tumors and 3 (4%) were grade III meningiomas. In turn, 29/78 (37%) samples showed a diploid cytogenetic profile, 26/78 (33%) had isolated monosomy 22/del(22q) and 22/78 (28%) tumors displayed a complex iFISH karyotype; the remaining case showed an isolated loss of chromosome 1p. All but 3 cases underwent complete tumor resection at diagnostic surgery. Adjuvant radiotherapy was given after surgery in 4 WHO grade II/III and 2 WHO grade I tumors. At the moment of closing this study, 6/75 patients had relapsed (8%) and one showed tumor regrowth (after partial tumor resection) after a median follow-up of 48 months (range: 1 to 238 months).

In this group of 78 freshly-frozen meningiomas, GEP analysis was performed in 40 samples and MFC immunophenotypic studies were done in 51 samples (13 cases were analyzed by both methods). Detailed information about the most relevant clinical, histopathological and cytogenetic characteristics of each of the tumor samples analyzed is provided in Table 4.

Table 4. Relevant clinical, histopathological, and genetic characteristics of the 78 meningioma samples studied by multiparameter flow cytometry immunophenotyping (n=38), gene expression profiling by oligonucleotide arrays (n=27) or both (n=13).

Tumor ID	iFISH Karyotype	Gender	Age	Histopathologic subtype (WHO)	Tumor grade	Localization	Edema	Tumor relapse	Analyses performed
8	Diploid	F	62	Atypical	II	Spinal	No	No	GEP
10	Diploid	F	62	Atypical	II	Spinal	No	No	GEP
14	Diploid	F	76	Transitional	I	Parasagittal	Light (+)	No	GEP
17	Diploid	F	73	Transitional	I	Cranial base	No	No	GEP
20	Diploid	M	56	Transitional	I	Cranial base	No	No	MFC/GEP
24	Diploid	F	68	Transitional	I	Cranial base	No	No	GEP
25	Diploid	F	63	Psammomatous	I	Cranial base	No	No	GEP
26	Diploid	F	84	Secretory	I	Convexity	Severe (+++)	No	GEP
27	Diploid	F	36	Transitional	I	Spinal	No	No	GEP
28	Diploid	F	68	Transitional	I	Cranial base	Severe (+++)	No	MFC/GEP
30	Diploid	F	69	Meningothelial	I	Cranial base	Light (+)	No	MFC/GEP
31	Diploid	M	77	Transitional	I	Cranial base	Moderate (++)	No	GEP
32	Diploid	M	54	Transitional	I	Cranial base	Moderate (++)	No	MFC/GEP
34	Diploid	F	42	Meningothelial	I	Tentorial	No	No	MFC
35	Diploid	F	47	Meningothelial	I	Cranial base	No	No	GEP
36	Diploid	M	65	Atypical	II	Cranial base	No	No	GEP
37	Diploid	F	54	Atypical	II	Cranial base	Moderate (++)	No	GEP
43	Diploid	F	42	Meningothelial	I	Convexity	Moderate (++)	No	MFC
45	Diploid	F	43	Meningothelial	I	Convexity	No	No	MFC
47	Diploid	F	42	Transitional	I	Convexity	Light (+)	No	MFC
51	Diploid	F	61	Secretory	I	Cranial base	Moderate (++)	No	MFC
52	Diploid	M	56	Rhabdoid	III	Convexity/Parasagittal	Severe (+++)	No	MFC
56	Diploid	F	63	Psammomatous	I	Cranial base	Severe (+++)	No	MFC
60	Diploid	F	54	Angiomatous	I	Tentorial	Light (+)	No	MFC
66	Diploid	F	61	Meningothelial	I	Cranial base	No	No	MFC
67	Diploid	M	30	Transitional	I	Parasagittal	No	No	MFC

Table 4. *(continued)*

69	Diploid	F	53	Meningothelial	I	Cranial base	No	No	MFC
72	Diploid	F	51	Meningothelial	I	Cranial base	No	No	MFC
74	Diploid	F	69	Psammomatous	I	Cranial base	Moderate (++)	No	MFC
1	Monosomy 22	F	76	Fibroblastic	I	Cranial base	No	Yes	GEP
3	Monosomy 22	F	72	Psammomatous	I	Spinal	No	No	GEP
6	Monosomy 22	F	77	Transitional	I	Parasagittal	No	No	GEP
12	Monosomy 22	F	59	Psammomatous	I	Spinal	No	No	GEP
15	Monosomy 22	M	54	Psammomatous	I	Cranial base	No	No	GEP
21	Monosomy 22	F	81	Psammomatous	I	Spinal	No	No	GEP
22	Monosomy 22	M	76	Transitional	I	Spinal	No	No	GEP
23	Monosomy 22	F	54	Psammomatous	I	Cranial base	No	No	MFC/GEP
29	Monosomy 22	F	74	Atypical	II	Spinal	No	No	GEP
33	Monosomy 22	F	34	Fibroblastic	I	Convexity	No	No	MFC/GEP
39	Monosomy 22	F	65	Psammomatous	I	Spinal	No	No	GEP
40	Monosomy 22	F	53	Fibroblastic	I	Convexity	Light (+)	No	MFC/GEP
41	Monosomy 22	F	66	Fibroblastic	I	Convexity	Moderate (++)	No	MFC
42	Monosomy 22	F	49	Transitional	I	Convexity	Light (+)	No	MFC
46	Monosomy 22	F	42	Transitional	I	Convexity/Parasagittal	No	No	MFC
48	del(22q)	F	58	Fibroblastic	I	Convexity/Parasagittal	Severe (+++)	No	MFC
49	Monosomy 22	F	75	Psammomatous	I	Spinal	No	No	MFC
50	Monosomy 22	F	78	Psammomatous	I	Spinal	No	No	MFC
54	Monosomy 22	F	56	Fibroblastic	I	Convexity/Parasagittal	Light (+)	No	MFC
55	Monosomy 22	F	57	Fibroblastic	I	Cranial base	No	No	MFC
57	del(22q)	M	58	Transitional	I	Parasagittal	Light (+)	Yes	MFC
58	Monosomy 22	F	66	Psammomatous	I	Convexity	No	No	MFC
63	Monosomy 22	F	69	Psammomatous	I	Spinal	No	No	MFC
65	Monosomy 22	F	66	Transitional	I	Parasagittal	No	No	MFC
70	Monosomy 22	M	77	Meningothelial	I	Cranial base	No	No	MFC
71	Monosomy 22	F	48	Fibroblastic	I	Parasagittal	No	No	MFC
2	-(1p/11/18/22) +(1q/7/17)	F	64	Atypical	II	Convexity	Moderate (++)	No	GEP
4A	-14 +(1q/22/X)	F	30	Anaplastic	III	Convexity	Moderate (++)	Yes	GEP
4B	-14 +(1q/22/X)	F	30	Papillary	III	Convexity	Moderate (++)	Yes	MFC
5A	-(1p/22)	M	73	Meningothelial	I	Cranial base	Light (+)	Yes	GEP
5B	-(1p/22)	M	75	Transitional	I	Cranial base	Light (+)	Yes	GEP
7	-(1p/14/22/Y) +(15/18)	M	23	Atypical	II	Parasagittal	Severe (+++)	No	GEP
9	-(1p/6/14/22)	M	41	Atypical	II	Convexity	Light (+)	Yes	MFC/GEP
11	-14 +(7/10/15/17) +18	F	35	Atypical	II	Convexity/Parasagittal	Severe (+++)	No	MFC/GEP
13	-(14/22/X)	F	76	Meningothelial	I	Convexity/Parasagittal	No	No	MFC/GEP
16A	-(1p/14/22) +9	F	60	Transitional	I	Convexity	Moderate (++)	Yes	MFC/GEP
16B	-(1p/14/22)	F	62	Atypical	II	Convexity	Moderate (++)	Yes	GEP
18	- (1/10/14/15/17/18/22/X)	F	52	Atypical	II	Convexity/Parasagittal	Moderate (++)	No	MFC/GEP
19	-(1p/14/22/X) +1q	F	70	Transitional	I	Cranial base	Moderate (++)	No	MFC/GEP
38	- (1p/10/14/22/18)	F	84	Meningothelial	I	Convexity	Severe (+++)	No	MFC
44	+(1q/7/10/15/17/18/22/Y/X)	M	68	Meningothelial	I	Convexity/Parasagittal	Moderate (++)	No	MFC
53	-(14/22/Y)	M	61	Meningothelial	I	Convexity	Light (+)	No	MFC
59	-(1p/7/14/15/18/22) +1q	F	76	Transitional	I	Convexity	Moderate (++)	No	MFC
61	-(1p/18/22) +9	M	54	Meningothelial	I	Convexity	No	No	MFC
62	+(1q/7/14/15/17) ++18	M	68	Transitional	I	Parasagittal	Severe (+++)	No	MFC
64	-(1p/22)	M	48	Transitional	I	Parasagittal	Severe (+++)	No	MFC
68	-(1p/22/Y) +1q	M	66	Transitional	I	Convexity	Moderate (++)	No	MFC
75	-(1p/14/18/22) +1q	F	72	Transitional	I	Convexity	Moderate (++)	No	MFC
73	-1p	M	66	Meningothelial	I	Intraosseous	No	No	MFC

WHO: World Health Organization; M: Male; F: Female; iFISH: interphase fluorescence *in situ* hybridization; Tumors with ≥2 chromosomal changes were classified as carrying complex karyotypes; GEP: gene expression profiling, MFC: multiparameter flow cytometry immunophenotyping.

Apart from the overall series of 78 tumor specimens corresponding to 75 meningioma patients, an additional group of 33 patients diagnosed with meningioma (11 males and 22 females; mean age of 66±14 years; range: 24 to 83 years; 32 WHO grade I and one WHO grade II/atypical meningioma) was analyzed by MFC for an additional specific marker (CD206) and included in the study later on (as described in chapter 5). Characterization according to the iFISH cytogenetic profile for this group of samples was as follows: 14/33 diploid, 5/33 monosomy 22/del(22q), and 14/33 complex karyotype meningiomas.

1.2. Patients and samples used for the construction of a risk-stratification model including cytogenetic and copy number (CN) alterations by SNP-arrays

In this part of the study, a larger number of meningioma samples from the University Hospital of Salamanca, which had been previously processed and stored at -150ºC, were included. Thus, a total of 302 patients (91 males, 211 females; mean age of 60±15 years, ranging from 16 to 87 years) were analyzed in this part of the study. From the histopathological point of view, 264 cases (87%) were WHO grade I tumors, 30 cases (10%) were grade II and 8 (3%) were grade III meningiomas. Around half of the tumors corresponded to meningothelial meningiomas (n=147; 49%), 57 were transitional (19%), 34 psammomatous (11%), 24 were atypical (8%), 17 fibroblastic (5%), 7 angioblastic (2%), 5 anaplastic (2%), 5 secretory (2%), 3 chordoid (1%) and, 2 rhabdoid (1%) meningiomas; the remaining case was a papillary tumor. According to tumor localization, most meningiomas (n=276; 91%) corresponded to intracranial tumors (cranial base, 35%; convexity, 21%; parasagittal, 18%; falcine, 12%; tentorial, 3%, and; intraventricular, 2%) and only 26 (9%) were spinal meningiomas. Brain edema was found in 173/302 (57%) patients and evaluated according to its extension as light in 72 cases (24%), moderate in 65 (21%) and severe edema in 36 cases (12%). The great majority of the patients (283/302; 94%) underwent complete tumor resection; this include 35/38 grade II/III tumors. In addition to surgery, adjuvant radiotherapy was given to 21 WHO grade II/III tumors. One patient with anaplastic meningioma also received systemic chemotherapy, in addition to RT.

At the moment of closing the study, 42/302 patients (14%) had relapsed after a median follow-up of 65 months. From the whole series, 41 cases were excluded from survival analyses because follow-up data was not available (n=30 cases) or the patient died shortly after surgery (n=11 cases). Follow-up studies were performed according to a standard clinico-biological protocol, which included MRI techniques performed 3 months after surgery and

every 12 months thereafter; whenever clinical signs and/or symptoms were noted and/or a relapse was suspected, additional MRI studies were performed.

From this series of 302 freshly-frozen meningiomas, a subset of 50 samples was analyzed by SNP-arrays (previously reported in the literature [118]) in order to validate the CN alteration profiles obtained by iFISH.

2. Interphase fluorescence *in situ* hybridization (iFISH) studies

For all tumor samples obtained after surgery, iFISH analyses were performed for the identification of the numerical alterations of those chromosomes and chromosomal regions more frequently altered in meningiomas, and further characterize the iFISH cytogenetic profile of the tumor cell. For that purpose, a panel of commercial fluorescently-labeled probes obtained from Vysis Inc. (Downers Grove, IL, USA), for 11 distinct chromosomes, was used in double-stainings, as previously described in detail [117]: for chromosomes 9 and 22, the LSI BCR/ABL dual-color probe was used; for chromosomes 15 and 17, the LSI PML/RAR-α dual-color probe was employed; for chromosomes 14 and 18, the LSI IgH/BCL2 dual-color probe; for chromosome 1, the 1p36/1q25 dual-color probe; CEP 7 and 10 DNA probes conjugated with Spectrum Orange (SO) and Spectrum Green (SG) were used for chromosomes 7 and 10, respectively, and; the CEP X (SO) and CEP Y (SG) probes were employed for chromosomes X and Y, respectively. Selection of the probes was based on the following criteria: specificity for a target chromosome and chromosomal region that is known to be frequently deleted and/or gained in meningiomas, and the quality of the probe, as evaluated by its hybridization specificity, efficiency and the fluorescence intensity of the hybridization signals obtained in normal diploid nuclei.

Once obtained, tumor cells were fixed in 3/1 methanol-acetic acid (vol/vol) and dropped onto cleaned, poly-L-lysine-coated slides (Sigma, St. Louis, MO, USA). The slides were then sequentially incubated for 10 minutes at 37°C with 0.1 mg/mL pepsin (Sigma), fixed in 1% acid-free formaldehyde (Merck, Darmstadt, Germany) for 10 minutes at room temperature, and dehydrated in increasing concentrations of ethanol (Merck) in water (70%, 90% and 100%). After this procedure, the slides containing DNA from both the cells and the probes were denatured at 75°C (6 minutes) and immediately hybridized overnight (38°C) in a Hybrite thermocycler (Vysis Inc). Once this incubation period was completed, slides were sequentially washed (5 minutes at 46°C) in 50% formamide (Merk) in a 2X saline sodium citrate (SSC) buffer and in 2X SSC (2 minutes at 46°C). Cells were then counterstained with 4,6-diamino-2-

phenylindole (DAPI) (0.1µg/mL) (Sigma); Vectashield (Vector Laboratories Inc, Burlingame, CA, USA) was used as anti-fading agent. The number of hybridization spots was evaluated using a BX60 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 100x oil objective; for each slide, at least 200 nuclei were evaluated. For all slides measured, the number of unhybridized cells in the areas assessed was irrelevant (<1%), and only those spots with a similar size, intensity and shape, were scored. Doublet signals were rarely found (< 2% of all nuclei/slide); if present, they were considered as a single spot. The criteria used to define presence of numerical abnormalities for each of the chromosomes analyzed was based on the study of normal control samples, as previously described in detail [117]. Briefly, gains and losses of specific chromosomal regions were considered to occur when ≥5% and ≥10% of the nuclei showed an increased and decreased number of fluorescent signals (spots) with respect to normal diploid cells, respectively.

3. Multiparameter flow cytometry immunophenotypic studies

For the multiparameter flow cytometry immunophenotypic analyses, 51 freshly-frozen tumor samples were thawed in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37ºC, following conventional procedures. In a subgroup of 18/51 samples, both fresh (processed <4h after surgery) and freshly-frozen tumor tissue samples were analyzed in parallel. In both cases, single tumor cell suspensions were obtained through conventional mechanical disaggregation procedures [377] in phosphate buffered saline (PBS) containing 10% FBS (Invitrogen), 1% bovine serum albumin (BSA) (Sigma) and 2mM EDTA (Merck). Meningioma cells were then stained for 30 minutes at 4ºC in the darkness, with a large panel of monoclonal antibodies (MAb) in 3-color combinations - pacific blue (PacB) / fluorescein isothiocyanate (FITC) / phycoerythrin (PE) -, and finally washed with a PBS with 10% FBS + 1% BSA + 2mM EDTA. In staining for cytoplasmic (Cy) markers, before incubation with the MAb, cells were incubated at -20ºC for 1h (freshly-frozen tissues) or overnight (fresh tissues) in a citrate buffer [250 mM sucrose (Sigma), trisodium citrate 40 mM (Sigma), and 5% (vol/vol) dimethylsulfoxide (DMSO; Merck), pH=7.6], as described elsewhere [378].

For the immunophenotypic analysis of meningioma cells, a panel was built with the following MAb: CD2-FITC, CD13-PE, CD14-PE, CD33-PE, CD58-PE, CD69-PE, HER2/neu-PE and HLA-DR-FITC, purchased from Becton/Dickinson Biosciences (BD, San Jose, CA, USA); CD22-FITC, CD37-FITC, CD53-PE, CD55-FITC, CD81-PE, CD99-PE, CD200-PE, EGFR-PE, IGFR-PE and PDGFRβ-PE from BD Pharmigen (San Diego, CA, USA); CD9-FITC, CD16-FITC, CD63-FITC and

HLA-I-FITC purchased from Beckman/Coulter (Hialeah, FL, USA); CD44-PE and CD59-FITC obtained from Immunostep SL (Salamanca, Spain); CD38-FITC, (Cy)Bcl2-FITC and (Cy)CD68-FITC were purchased from Cytognos SL (Salamanca, Spain), DAKO (Glostrup, Denmark) and An der Grub (ADG, Vienna, Austria), respectively (Table 5). All MAb combinations systematically contained the DRAQ5 DNA dye (Cytognos SL) and CD45-PacB (DAKO), for reproducible identification of nucleated cells and leukocytes in the sample, respectively. For the specific identification of lymphocyte subsets, an additional 5-color staining was performed: CD45 pacific orange (PacO; Invitrogen), CD3-PacB (BD Pharmingen), CD8-FITC (BD), CD19-FITC (BD) and CD56-PE (Cytognos SL). In a subset of 12/51 samples, regulatory T cells (Tregs) and co-stimulatory molecules were analyzed using CD4-PacB (BD Pharmingen), CD25-PE (BD), CD127-FITC (BD Pharmingen) and CD28-PE (BD); $CD4^{+}CD25^{hi}CD127^{-/lo}$ Tregs were detectable at a frequency of 1 Treg cell in 30.000 cellular events acquired.

In the additional series of 33 freshly-obtained meningioma samples (see chapter 5), cells were also stained for a MAb combination targeting the evaluation of the CD206 M2-macrophage marker (BD Pharmingen) using a 5-color staining - HLA-DR-PacB / CD45-PacO / CD206-FITC / CD14-PE / DRAQ5 -, as well as for the 5-color stainings defined above, targeting the identification of lymphocyte subsets and Tregs; in a subgroup of 5/33 samples, the CD163-PE MAb (BD Pharmingen) was also included in this part of the analysis.

To assess control baseline autofluorescence levels, an aliquot of each tumor sample stained only for DRAQ5 was measured in parallel. Staining for DRAQ5 was performed 5 min prior to the measurement in the flow cytometer [379]. Absence of blood infiltration of the tumor single cell suspensions was confirmed based on the lack of $CD16^{+}CD45^{+}$ neutrophils in the sample. Data acquisition was performed for $\geq 1x10^{5}$ cells per antibody combination in a FACSCanto II flow cytometer (BD), using the FACSDiva 6.0 software (BD). The INFINICYT software (Cytognos SL) was used for data analysis devoted to the evaluation of the percentage of positive cells and of the amount of protein expression per cell [mean fluorescence intensity (MFI) expressed in arbitrary units scaled from 0 to 262,144] for each individual marker within a cell population. An antigen was considered to be positive when the percentage of positive cells was >20% or the MFI exceeded the mean MFI+3 standard deviations (SD) of the baseline autofluorescence levels obtained for the unstained cells. The proliferation index (PI) of tumor cells was calculated as the percentage of cells showing a higher DNA content than that of G0/G1 cells, after excluding debris and cell doublets in FSC-Area *vs.* SSC-Area and DRAQ5-Area *vs.* DRAQ5-Width bivariate dot plots, respectively [380].

Table 5. Antibody reagents used for the immunophenotypic analysis of meningiomas.

Specificity	Antibody	Source	Clone
Major histocompatibility complex (MHC) molecules	***HLA-I - FITC***	IOTest [1]	B9.12.1
	HLA-DR - FITC	BD [2]	L234
Complement regulatory proteins	***CD55 - FITC***	BD Pharmingen [3]	IA10
	CD59 - FITC	Immunostep [4]	VJ1/12.2
Tetraspanins	***CD9 - FITC***	IOTest [1]	ALB6
	CD37 - FITC	BD Pharmingen [3]	M-B371
	CD53 - PE	BD Pharmingen [3]	HI29
	CD63 - FITC	IOTest [1]	CLBGran/12
	CD81 - PE	BD Pharmingen [3]	JS-81
Other cell adhesion molecules	***CD2 - FITC***	BD [2]	S5.2
	CD22 - FITC	BD Pharmingen [3]	HIB22
	CD44 - PE	Immunostep [4]	HP2/9
	CD56 - PE	Cytognos [5]	C5.9
	CD58 - PE	BD [2]	L306.4
	CD99 - PE	BD Pharmingen [3]	TÜ12
Ectoenzymes	***CD13 - PE***	BD [2]	L138
	CD38 - FITC	Cytognos [5]	LD38
Anti-apoptotic proteins	***Bcl2 - FITC***	DAKO [6]	124
Growth factor receptors	***EGFR - PE***	BD Pharmingen [3]	EGFR.1
	HER2/neu - PE	BD [2]	Neu 24.7
	IGFR - PE	BD Pharmingen [3]	1H7
	PDGFR - PE	BD Pharmingen [3]	28D4
Scavenger and mannose receptors	***CD163 - PE***	BD Pharmingen [3]	GHI/61
	CD206 - FITC	BD Pharmingen [3]	19.2
Other molecules	***CD3 - PacB***	BD Pharmingen [3]	UCHT1
	CD4 - PacB	BD Pharmingen [3]	RPA-T4
	CD8 - FITC	BD [2]	SK1
	CD14 - PE	BD [2]	MϕP9
	CD16 - FITC	IOTest [1]	3G8
	CD19 - FITC	BD [2]	4G7
	CD25 - PE	BD [2]	2A3
	CD28 - PE	BD [2]	L293
	CD33 - PE	BD [2]	P67.6
	CD45 - PacB	DAKO [6]	T29/33
	CD45 - PacO	Invitrogen [7]	HI30
	CD68 - FITC	ADG [8]	Ki-M7
	CD69 - PE	BD [2]	L78
	CD127 - FITC	BD Pharmingen [3]	HIL-7R-M21
	CD200 - PE	BD Pharmingen [3]	MRC OX-104

[1] IOTest (Beckman Coulter, Brea, CA, USA);
[2] BD Biosciences (BD, San José, CA, USA);
[3] BD Pharmingen (San Diego, CA, USA);
[4] Immunostep (Immunostep SL, Salamanca, Spain);
[5] Cytognos (Cytognos SL, Salamanca, Spain);
[6] Dako (Dako, Glostrup, Denmark);
[7] Invitrogen (Invitrogen, Carlsbad, CA, USA);
[8] An der Grug (ADG, Kaumberg, Austria).

3.1. Fluorescence activated cell sorting (FACSorting) and morphologic/genetic characterization of the FACS-purified cell populations

Purification of different cell populations coexisting in meningioma samples was performed in 12 freshly-obtained tumor samples using a 4-way fluorescence activated cell sorter (FACSAria; BD) and the FACSDiva software (see chapter 4). Prior to sorting, cells were stained with CD45-PacB/HLA-DR-FITC/CD44-PE/DRAQ5, as described above. Four different nucleated cell populations ($DRAQ5^{hi}$) were isolated (purity>90%; mean: 96%±3%, 94%±4%, 98%±1% and 97%±2%, respectively) based on the following phenotypes: i) $SSC^{lo}CD45^{hi}CD44^{hi}$; ii) $SSC^{lo}CD45^{+}HLA\text{-}DR^{hi}CD44^{-}$; iii) $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ and iv) $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$. The four sorted cell populations were placed in both methanol/acetic 3/1 (vol/vol) for further iFISH analyses with the 9p34/22q11.2 dual color probe (Vysis Inc.), as described above, and the PreservCyt solution (Cytyc Corporation, Boxborough, MA, USA) employed for morphological studies. For the latter studies, slides were prepared using the ThinPrep 5000 (Cytyc Corporation) automated slide processor, stained with the Papanicolau stain using the Shandon Varistain Gemini automated instrument (Thermo Fischer Scientific Inc., Waltham, MA, USA), and analyzed in an Olympus BX5 microscope equipped with a 100x oil objective.

3.2. Phagocytic and endocytic studies

The phagocytic activity of the different cell populations present in the tumor (n=5) was evaluated through their ability to uptake FITC-conjugated *E. coli*, using the PHAGOTEST reagent kit (Orpegen Pharma, Heidelberg, Germany) [381]. In parallel, the endocytic capacity of the same cell populations was investigated in another group of tumors (n=7) through the ability of cells to capture antigens at 37°C *vs.* 4ºC (control), using a conventional dextran-FITC (Sigma-Aldrich) uptake assay [382]. For both phagocytic and endocytic assays, samples were counterstained with CD45-PacO, HLA-DR-PacB, CD44-PE and DRAQ5, to allow identification of the different cell subpopulations present in the sample. For data acquisition and analysis, the FACSCanto II flow cytometer was used following the protocol described above. Evaluation of the phagocytic/endocytic activities of each cell population was based on both the measurement of the percentage of phagocyting/endocyting cells (green fluorescence positive cells, using the control sample as reference) and their MFI, the latter values correlating with the number of bacteria/molecule levels internalized, per individual cell (see chapter 4).

4. Immunohistochemical analyses

In a representative subgroup of 12 samples, immunostaining of macrophages in tumor tissue sections was performed with the anti-CD68 antibody to confirm tissue localization of these cells (see chapter 5). For this purpose, 3µm-thick tissue sections were cut from paraffin-embedded blocks, deparaffinized, and stained in a Leica-BOND-III automated immunostainer (Leica Biosystems, Wetzlar, Germany) using the Bond Polymer Refine Detection kit (Leica Biosystems), according to the manufacturer's instructions. Briefly, after rehydrated, antigen retrieval was achieved with citrate buffer (pH=6.0) and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Sections were then incubated with an anti-CD68 mAb (clone KP1, dilution 1:50; Master Diagnóstica, Granada, Spain), for 20 minutes, followed by the rabbit anti-mouse Post Primary antibody reagent, the polymeric horseradish peroxidase (HRP) conjugated reagent and its 3,3'-diaminobenzidine (DAB) substrate, as the final chromogen. All immunostained sections were lightly counterstained with hematoxylin. Analysis was performed in an Olympus BX5 microscope equipped with a 100x oil objective.

5. Gene expression profiling (GEP) studies

In a subset of 40 meningioma cases, the GEP of freshly-frozen tumor tissue samples was analyzed with the Human Genome 133A Affymetrix array (Affymetrix Inc, Santa Clara, CA, USA). After thawing, tumors (fragments of 50-100 mg) were homogenized with a Potter-'S'-Elvehjem homogenizer (Uniform, Jencons, UK) in 1mL of TRIzol (Invitrogen). The homogenized TRIzol tumor, with 200 µl of chloroform, was then vigorously vortexed, incubated for 2-3 minutes at room temperature, and centrifuged at 12.000 x *g* (15 minutes at 4°C) to split up the different phases (a lower red phenolchloroform phase, an interphase, and a colorless upper aqueous phase). RNA was extracted from the upper aqueous phase, which was slightly and carefully removed, mixed with isopropanol, incubated for 15-30 minutes on ice, and centrifuged at 16.000 x *g* (10 minutes at 4° C) for RNA precipitation. After centrifugation, the supernatant (isopropanol) was removed and the pellet (RNA precipitate) mixed with 75% ethanol (in Diethylpyrocarbonate (DEPC)-treated water; Ambion Life Technologies, Carlsbad, CA, USA), and centrifuged at 12.500 x g (5 minutes at 4°C); the ethanol was then removed and the pellet dissolved in 50 µl DEP-treated water. The RNA concentration and quality were analyzed in an ND-1000 NanoDrop (NanoDrop Tecnologies Inc., Wilmington, DE, USA) (criterion for an acceptable quality of the RNA samples was based on an A260/A280 ratio >1.6).

Total RNA was further purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Briefly, the diluted RNA was mixed with the RLT buffer (lysis buffer from the QIAGEN kit; 2000 µl), β-mercaptoethanol (20 µl) and absolute ethanol (250 µl), transferred to an RNeasy column and centrifuged at 8.000 x *g* (1 minute). Next, the RPE-Ethanol buffer (from the QIAGEN kit; 500 µl) was added to the column and sequentially centrifuged at 8.000 x *g* (twice) and at 20.000 x *g* (2 minutes). Finally, an RNA-free treated tube was put under the column to receive the 30 µl of H20 RNase-free solution supplied with the kit, after centrifuging at 8.000 x *g* for 1 minute. The integrity/purity of the purified RNA was determined using a microfluidic electrophoretic system (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA, USA). Then, GEPs were analyzed according to the manufacturer's instructions, using the one-cycle cDNA synthesis kit and the Poly-A RNA gene chip control kit (Affymetrix Inc.). Data files containing data about the expression levels for the 40 tumors were normalized - Robust microarray normalization (RMA) - and analyzed using the R (version 2.7.0; http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) software tools. The microarray dataset is available in the Gene Expression Omnibus (GEO) public data repository (GSE43290 access code). Differentially expressed genes between samples from the different cytogenetic subgroups of meningiomas were identified using a supervised two-class unpaired Significance Analysis of Microarray (SAM) [383], based on a combined cutoff with a false discovery rate of <0.05 (T-test).

Further investigation of the altered pathways was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA). Through the IPA software, the specific cell functions associated with those genes under- or over-expressed in each specific cytogenetic subgroup of meningiomas, were first investigated. In a second step, the IPA software was used for a more detailed analysis of those signaling pathways involving genes which were under- and/or over-expressed among meningiomas with isolated monosomy 22/del(22q) vs other meningioma tumors (see chapter 5). For this purpose, those genes which were associated with the highest scored 'Bio Functions' for this specific cytogenetic subgroup of meningiomas, were selected (e.g. cell growth and proliferation of immune cells; hematological system development and function; immune cell trafficking; cell-to-cell signaling and interaction of immune cells; inflammatory response). In the following step, those genes showing more 'Direct Relationships', as well as those associated with 'Antigen Presenting Cell functions', were selected for the 'Path Designer' tool of the IPA software.

6. Copy number (CN) alterations by single-nucleotide polymorphism (SNP)-arrays

To validate the cytogenetic data obtained by iFISH in a high-throughput manner, CN alterations were evaluated by SNP-arrays in a subset of 50/302 samples (see chapter 7), previously reported by our group [118].

6.1. Isolation of tumoral and peripheral blood (PB) DNA

DNA from paired (freshly-frozen) tumor tissue and normal PB samples was purified using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. PB samples were initially centrifuged at 2.500 x *g* (10 minutes at room temperature) to separate the intermediate layer of leukocyte-enriched mononuclear cells from the plasma and the erythrocytes. This leukocyte layer was then extracted and incubated with a mixture of 20 µl of proteinase K, 4 µl of RNAse A (100 mg/ml) and 200 µl of the AL buffer (lysis buffer from the Kit; 10 minutes at 56°C). Regarding tumor samples, the tissue was initially incubated with 180 µl of the ATL buffer (tissue lysis buffer from the Kit) and 20 µl of proteinase K at 56°C (until cells were completely lysated), followed by an incubation with 4 µl RNAse A (2 minutes at room temperature), and then 200 µl of the AL buffer (10 minutes at 70°C). From this step on, all samples were processed in a similar way. Accordingly, they were placed in a QIAamp Spin Column with different solutions and centrifuged 10.000 rpm (1 minute at room temperature) until all sample volume had passed through the column: first, ethanol (200 µl), followed by the AW1 buffer (wash buffer from the Qiagen kit; 500 µl), and finally the AW2 buffer (wash buffer from the Qiagen kit; 500 µl). At each time, a clean collection tube was used to replace the tube containing the filtrated sample. To obtain the extracted DNA, the sample was incubated with 200 µl of "Low EDTA" buffer [10 mM Tris (tris(hydroxymethyl)aminomethane); 0.1 mM EDTA] for 5 minutes (room temperature), and centrifuged at 10.000 rpm (1 minute at room temperature) to pass through the column one last time. DNA purity and integrity were determined with a NanoDrop-1000 spectrophotometer and by conventional electrophoretic procedures in 1% agarose gel, respectively.

6.2. SNP-array hybridization and analysis

For SNP-array hybridization, the GeneChip Human Mapping 500K array set (250K Nsp and 250K Sty arrays; Affymetrix Inc.), which provides information about >500.000 SNPs, was used according to the manufacturer's instructions. Briefly, total DNA was digested with restriction enzymes and ligated to the corresponding adaptors, following conventional Affymetrix procedures (Affymetrix Inc.). A generic primer was used in triplicate to amplify adaptor-ligated DNA fragments, through a polymerase chain reaction (PCR). After hybridization with the sample's DNA, the chips were washed, labeled with streptavidin-phycoerythrin and scanned using a GeneChip Scanner 3000 (Affymetrix Inc.). The SNP call rate per array was always ≥92% (range: 92% to 99.8%). Overall, 200 '.CEL' files containing data on the SNP-arrays (one for each type of the 250K chips Nsp and Sty) for each type of sample (paired tumor and PB DNA), were obtained. For the analysis of SNP-array data, the GCOS (version 1.3, Affymetrix), the Copy Number Analysis Tool (CNAT v4.0, Affymetrix), dChip 2007 (http//www.dchip.org; Dana Farber Institute, Boston, MA) and the GeneChip Genotyping Analysis (GTYPE 4.1; Affymetrix) software programs, were used; CN values were calculated for each SNP and plotted according to chromosomal localization. Genotypes were generated using the BRLMM algorithm included in the Genotyping Console software (version 3.0.2; Affymetrix). Based on the results obtained on normal PB samples, cutoff values of ≤1.30 and ≥2.50 (arbitrary units) were used to establish CN losses and gains, respectively.

6.3. Validation series

In order to further confirm our findings, another external series of 82 patients with meningioma, which had been reported by others [75], and whose tumors had been analyzed by SNP-arrays (100K Affymetrix SNP arrays), with the corresponding '.CEL' files together with additional information about tumor characteristics and patient survival being available at the GEO public database (access code: GSE16583), were also included in this part of the study (in addition to our 50 tumors analyzed by SNP-arrays). From these patients, follow-up data was available in 108/132 and 17 of them (13%) had relapsed after a median follow-up of 59 months (range: 1 to 115 months).

7. Statistical methods and hierarchical clustering

For each continuous variable analyzed, its median, mean and standard deviation (SD) values, as well as range and both the 25^{th} and 75^{th} and the 10^{th} and 90^{th} percentiles, were calculated; for categorical variables, frequencies were reported. Statistical significance was determined through the non-parametric Kruskal-Wallis and Mann-Whitney U tests (for continuous variables) or the Pearson´s Chi-square test (for categorical variables); the Spearman's correlation was used to explore the degree of correlation between different variables. The most discriminant cut-off value for low *vs.* high tumor infiltration by immune cells was calculated using receiver operating characteristic (ROC) curve analysis. The Kaplan-Meier method was used to construct relapse-free survival (RFS) curves, and the (one-sided) log-rank test was applied to compare RFS curves. For multivariate analysis of patient RFS, the Cox stepwise regression model was used. In this part of the study, only those variables showing a significant association with RFS in the univariate analysis were included. For all statistical analyses, the SPSS software package was used (version 15.0; SPSS Inc., Chicago, IL, USA). P-values <0.05 (with an FDR correction for multiple comparisons of <10%) were considered to be associated with statistical significance.

For unsupervised clustering analyses, normalization of the datasets was performed for each parameter by calculating the ratio between the value obtained for each sample and the median of all samples analyzed. A logarithmic (base 2) transformation was applied to the ratio values, and the log2 ratios were then used for hierarchical clustering analyses (Cluster 3.0 and Tree View software; Stanford University, Stanford, CA, USA). Unsupervised hierarchical cluster analyses were performed using the Pearson correlation and the average linkage clustering method. Principal component (PC) analysis (PCA) was performed using the MultiExperiment Viewer Software (MeV v4.8, TM4 Microarray Software Suite, Boston, MA, USA).

Chapter IV

Immunophenotypic identification and characterization of tumor cells and infiltrating cell populations in meningiomas

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1. Abstract

Meningiomas are primary tumors of the central nervous system composed of both neoplastic and other infiltrating cells. Here, we determined the cellular composition of 51 meningioma samples by multiparameter flow cytometric (MFC) immunophenotyping, and investigated the potential relationship between mRNA and protein expression levels of neoplastic cells. For immunophenotypic, morphologic and cytogenetic characterization of individual cell populations, a large panel of markers was used together with phagocytic/endocytic functional assays and MFC sorting. Overall, our results show coexistence of $CD45^-$ neoplastic cells and $CD45^+$ immune infiltrating cells in all meningiomas. Infiltrating cells included tissue macrophages, with an $HLA\text{-}DR^+CD14^+CD45^+CD68^+CD16^{-/+}CD33^{-/+}$ phenotype and high phagocytic/endocytic activity, and a small proportion of cytotoxic lymphocytes (mostly T $CD8^+$- and NK-cells). Tumor cells showed expression of multiple cell adhesion proteins, tetraspanins, HLA-I/HLA-DR molecules, complement regulatory proteins, cell surface ectoenzymes and growth factor receptors. Noteworthy, the relationship between mRNA and protein levels was variable, depending on the proteins evaluated and the level of infiltration by immune cells. In summary, our results indicate that MFC immunophenotyping provides a reliable tool for the characterization of the patterns of protein expression of different cell populations coexisting in meningioma samples, with a more accurate measure of gene expression profiles of tumor cells at the functional/protein level than conventional mRNA microarray, independently of the degree of infiltration of the tumor by immune cells.

2. Introduction

Meningiomas are primary tumors of the Central Nervous System (CNS) derived from the meningeal coverings of the spinal cord and the brain [6]. Although the tumor itself is mainly composed of neoplastic cells, presence of infiltrating inflammatory and normal residual/reactive cells (e.g. macrophage/microglial cells and lymphocytes), as detected by immunohistochemistry, have long been reported in meningioma tissue specimens [272, 274-276, 348, 384]. Infiltrating inflammatory cells are involved in the pathogenesis of multiple different tumors where they may be associated with a unique clinical behavior [272, 276, 385]. In turn, their presence may hamper precise evaluation of tumor cell-specific alterations, particularly quantitative assessment of their biochemical and molecular features (e.g. RNA or protein expression levels), due to variable numbers of infiltrating inflammatory cells in the sample [386, 387].

Because of the above limitations, in recent years techniques enabling isolation of individual cell populations from heterogeneous and complex tumor tissues (e.g. laser capture microdissection) are more frequently applied. However, these strategies do not allow isolation of large numbers of cells, they are not compatible with live cell analyses, and the limited amount of nucleic acids and other cell components obtained from microdissected samples limits their direct usage for high-throughput molecular studies such as microarray-based gene analysis [388-390]. Alternatively, multiparameter flow cytometry (MFC) immunophenotyping is a well-suited method for simultaneous identification, characterization and isolation of different cell populations in a sample, purified live cells being placed in a single cell suspension. In addition, MFC allows quantitative evaluation of protein expression levels in large numbers of individual cells, with highly reproducible and statistically reliable results [361]. Consequently, MFC emerges as an attractive tool for objective evaluation of the cellular composition of tumor samples, assessment of protein expression profiles (PEP) of both purified tumor and reactive/inflammatory cells and determination of the clinical impact of such inflammatory infiltrates.

The number of reported MFC immunophenotypic studies of meningiomas is very limited, and these studies are typically restricted to the analysis of the expression of a few individual markers for the whole sample cellularity. Among other markers, such studies reported expression of the CD44 cell adhesion molecule related to tumor invasion and metastasis [375, 376] and of CD68, a monocyte/macrophage associated marker, which could potentially be expressed by the tumor cells but also by infiltrating inflammatory cells [274]. To the best of our knowledge, no study has been reported so far in which the relationship

between the microarray gene expression profiles (GEP) and the proteins coded by the affected genes has been specifically evaluated for meningioma tumor cells.

Here, we used MFC to analyze the cellular composition and phenotype of 51 meningiomas for a broad set of proteins. In a subset of samples we further evaluated both the impact of freezing on the PEP of tumor cells, and the relationship in individual samples between the amount of mRNA and the corresponding protein levels. Overall, our results show that meningiomas systematically display infiltration by inflammatory cells (mainly tissue macrophages) among a major but variable percentage of neoplastic cells. The PEP of tumor cells was not significantly affected in frozen *vs.* fresh tumor samples, whereas the relationship between mRNA and protein levels was variable depending on the specific proteins evaluated.

3. Results

3.1. Immunophenotypic identification and characterization of meningioma cell populations

Immunophenotypic analysis of meningioma samples (n=51) systematically showed the presence of multiple cell populations, which included both reactive/inflammatory and neoplastic cells (Figure 11). Expression of CD45 was restricted to around one fourth of the cells (24±20%), whereas most cells in the tumor samples (76%±20%) corresponded to $CD45^-$ neoplastic cells (Figure 11B). Infiltrating $CD45^+$ inflammatory cells included two distinct populations (Figure 11C). The first one showed a SSC^{lo} and $CD45^{hi}$ phenotype (Figure 11C-11E), compatible with that of $CD3^+$ T cells (1.4%±1.5% of the cells, Figure 11G) - mostly $CD8^+$ (1.1%±1.3%) - and $CD3^-/CD19^-/56^+$ NK cells (0.2%±0.3% of the cells, Figure 11H). Of note, $CD4^+CD25^{hi}CD127^{-/lo}$ Treg cells were only found in 4/12 cases analyzed, where they represented 5%±4% of all $CD4^+$ T cells. Expression of the CD28 co-stimulatory molecule was detected in 32%±23% and 62%±24% of the $CD8^+$ and $CD8^-$ T cells, respectively. B cells were detected in 58% of the tumors at very low frequencies (0.03%±0.05% of the cells). The second population of $CD45^+$ cells showed surface membrane (Sm) reactivity for HLA-DR^+, $CD14^+$ and $CyCD68^+$ (22%±18% of the overall cellularity) and variable positivity for CD16 (47±20%) and CD33 (39±32%), an immunophenotype consistent with that of tissue macrophages (Figure 11C, 11I and 11J). These latter $CD45^+$HLA-$DR^+CD14^+CD16^{-/+}CD33^{-/+}$ cells systematically expressed the CD9, CD53, CD63 and CD81 tetraspanin molecules, the CD55 and CD59 complement regulatory proteins and HLA-I. The CD38 ectoenzyme, the CD2 and CD44 cellular adhesion proteins and the bcl-2 anti-apoptotic protein were also detected in all cases, although in variable percentages of these cells (Table 6). In turn, expression of CD13, CD99, CD58, CD22, CD69 and CD37 was detected in these $CD45^+$HLA-DR^+CD14^+ cells from only a subgroup of tumors (Table 6). The EGFR, IGFR and HER2/neu growth factor receptors and the CD200 protein were constantly negative in this cell population. In addition, these cells showed both a significant phagocytic (p=0.009) and endocytic (p=0.002) activity at 37ºC (Figure 12). Interestingly, no significant differences were found regarding the distribution of inflammatory cells and their subsets according to the degree of brain edema and the distinct histological subtypes (data not shown).

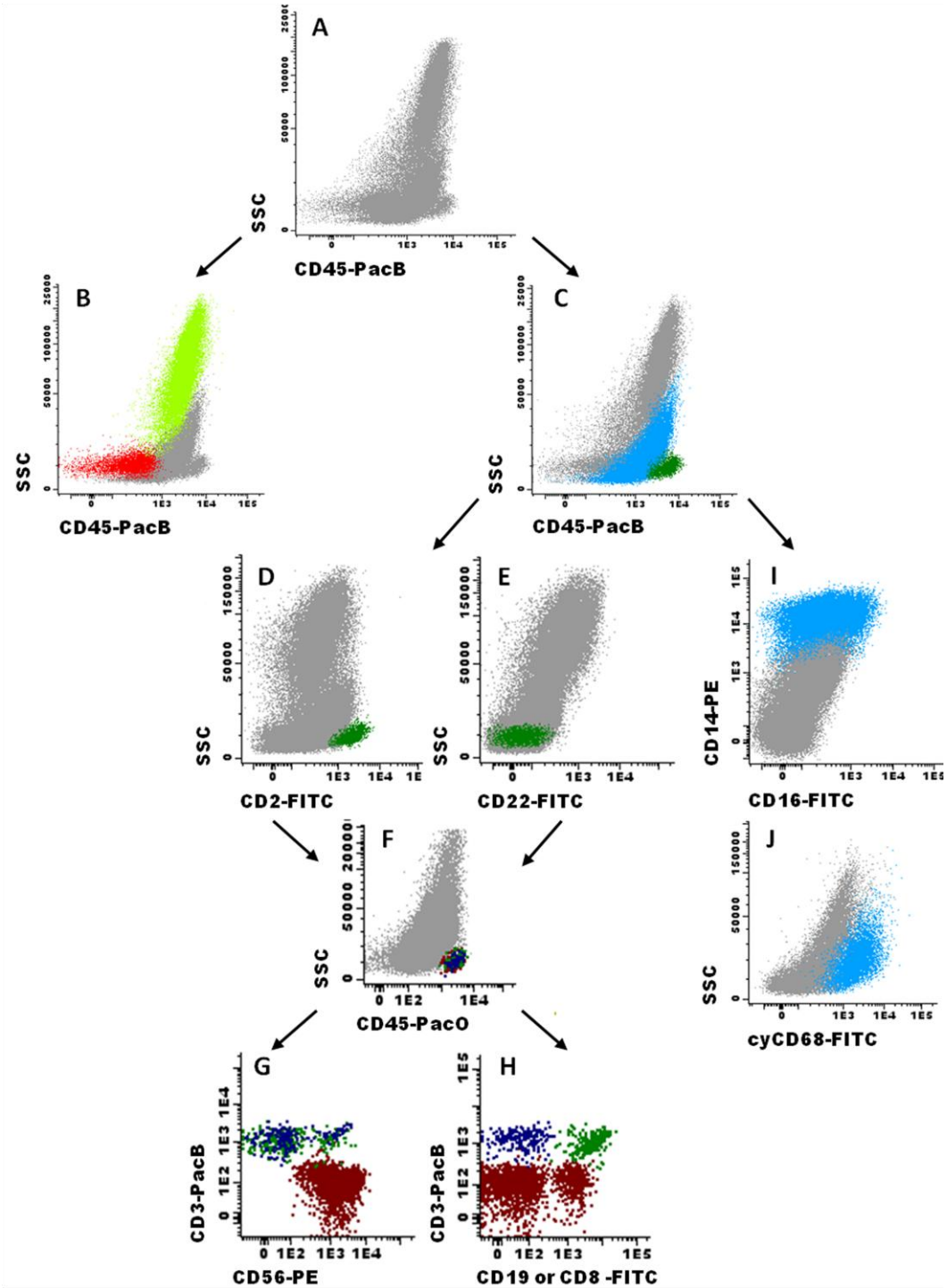

Figure 11. **Multiparameter flow cytometry immunophenotypic identification of different cell compartments in meningioma tissue samples.** Panel A shows the overall sideward light scatter (SSC) *vs.* CD45 pattern of reactivity for all nucleated cell compartments (DRAQ5^{+}) in a representative single cell suspension from a meningioma tissue specimen. Meningeal tumor cells (red and green dots) and CD45^{+} inflammatory cells infiltrating the tumor (blue and dark green dots) are shown in panels B and C, respectively; these latter infiltrating CD45^{+} cells corresponded to: i) lymphocytes expressing CD2 in the absence of CD22 (dark green dots in panels D and E), composed of CD8^{+} (dark green dots in panels F-H) and CD8^{-} (dark blue dots in panels F-H) T-lymphocytes plus NK cells (dark red dots in panels F-H), and; ii) CD45^{+} HLA-DR^{hi} CD44^{het} antigen presenting cells (blue dots in panels C, I and J) showing a CD14^{+} CD68^{+} CD16$^{-/+}$ immunophenotype.

Table 6. Patterns of protein expression of neoplastic cells and antigen-presenting (inflammatory) cells infiltrating the tumor in meningiomas (n=51).

Protein	Meningeal-tumor cells ($SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells)		Antigen presenting inflammatory cells ($CD45^{+}HLA\text{-}DR^{hi}CD14^{+}CD16^{-/+}$)	
	No. of positive samples / total samples (%)	% of positive cells *	No. of positive samples / total samples (%)	% of positive cells *
CD9	51/51 (100)	97 ± 10	18/18 (100)	99 ± 3
CD44	51/51 (100)	80 ± 26	18/18 (100)	66 ± 18
CD55	51/51 (100)	96 ± 12	18/18 (100)	98 ± 4
CD59	51/51 (100)	99 ± 2	18/18 (100)	100 ± 1
CD63	51/51 (100)	90 ± 15	18/18 (100)	87 ± 11
CD81	51/51 (100)	98 ± 5	18/18 (100)	98 ± 3
HLA-I	51/51 (100)	90 ± 14	18/18 (100)	100 ± 0
CD13	51/51 (100)	89 ± 15	15/18 (83)	63 ± 24
HER2/neu	49/51 (96)	73 ± 26	0/18 (0)	-
IGFR	49/51 (96)	73 ± 25	0/18 (0)	-
EGFR	40/51 (78)	69 ± 24	0/18 (0)	-
CD200	30/51 (59)	46 ± 22	0/18 (0)	-
CD38	44/51 (86)	66 ± 27	18/18 (100)	81 ± 22
Bcl2	44/51 (86)	65 ± 24	18/18 (100)	64 ± 26
HLA-DR	41/51 (80)	69 ± 23	18/18 (100)	98 ± 3
CD14	39/51 (76)	76 ± 19	18/18 (100)	99 ± 1
CD53	32/51 (63)	62 ± 24	18/18 (100)	95 ± 8
CD2	11/51 (22)	57 ± 24	18/18 (100)	79 ± 25
CD58	30/51 (59)	61 ± 22	13/18 (72)	81 ± 15
CD99	24/51 (47)	60 ± 21	14/18 (78)	53 ± 20
CD45	0/51 (0)	-	18/18 (100)	92 ± 7
CD16	0/51 (0)	-	13/18 (72)	47 ± 20
CD22	0/51 (0)	-	8/18 (44)	51 ± 19
CD69	0/51 (0)	-	7/18 (39)	24 ± 3
CD37	0/51 (0)	-	5/18 (28)	30 ± 5

* Results expressed as mean ± standard deviation (SD).

$CD45^{-}$ tumor cells displayed variable light scatter, HLA-DR and CD44 fluorescence levels and they consisted of two clearly defined subsets: $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ (23%±23%) and $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ (53%±24%) events. The latter cell population systematically displayed high reactivity in all cells for the CD9, CD63 and CD81 tetraspanin molecules, the CD55/CD59 complement regulatory proteins, HLA-I and the CD13 ectoenzyme. Other proteins which were expressed by this cell population in the majority of meningiomas (partial expression) were the IGFR, HER2/neu and EGFR growth factor receptors, CD14, CD38 and bcl-2 (Table 6). In addition, other proteins like CD53, CD58, CD200, CD99 and CD2 were only present in a subset of cells from a lower percentage of cases, while CD16, CD22, CD37 and CD69 were systematically

negative (Table 6). $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ tumor cells showed no detectable phagocytic activity, while they displayed an endocytic activity similar to that of tissue macrophages (Figure 12). Additionally, a significant correlation was found between the percentage of $SSC^{lo}CD45^{+}HLA\text{-}DR^{hi}CD44^{het}$ inflammatory cells and both the amount of expression of HLA-DR (r^2=0.4, p=0.001) and CD14 (r^2=0.4, p=0.001) and the percentage of neoplastic cells which were positive for these two markers (r^2=0.3, p=0.02 and r^2=0.4, p=0.005, respectively). As discussed below, $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ tumor cells showed absence of expression of all markers evaluated.

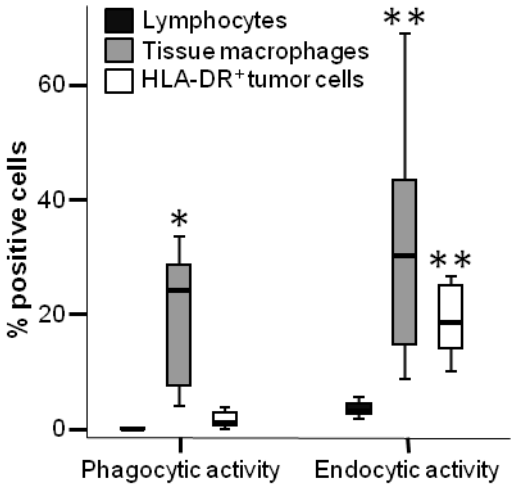

Figure 12. Phagocytic and endocytic ability of neoplastic tumor cells and different subpopulations of tumor infiltrating inflammatory cells. The phagocytic and endocytic activity (expressed as percentage of positive cells determined by the uptake of *E.coli*-FITC and dextran-FITC, respectively) of lymphocytes (black bars; negative control), tissue macrophages (grey bars) and tumor cells (white bars) is compared. *p=0.009; **p=0.002

Noteworthy, among $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells a significant correlation ($r^2 \geq 0.5$; $p \leq 0.02$) was found between the mean amount of expression of each protein/cell in paired fresh and freshly-frozen tissue samples (n=18), except for Cybcl2, CD2 and CD200 (Table 7). Despite such correlation, significantly higher levels were observed for freshly frozen cells for CD2, CD14, CD53, CD55, CD63, CD99 and HLA-DR ($p<0.05$; Table 7).

Table 7. Correlation between the amount of expression - mean fluorescence intensity (MFI) - of different proteins in tumor cells from paired fresh and freshly frozen meningioma tissue samples (n=18).

Marker	Cell localization	Mean amount of protein (MFI)/cell			Correlation coefficient	
		Fresh tissue *	Frozen tissue *	P-value	r^2	P-value
CD14	membrane	481 ± 406	655 ± 542	0.03	0.9	<0.001
CD99	membrane	186 ± 146	258 ± 247	0.03	0.9	<0.001
CD58	membrane	258 ± 217	354 ± 334	NS	0.9	<0.001
CD13	membrane	8771 ± 8733	9539 ± 10488	NS	0.8	<0.001
HLA-I	membrane	8040 ± 6294	7715 ± 5575	NS	0.8	<0.001
CD9	membrane	24043 ± 20352	27243 ± 22448	NS	0.8	<0.001
HLADR	membrane	662 ± 658	1482 ± 1564	0.001	0.7	<0.001
EGFR	membrane	473 ± 571	388 ± 367	NS	0.7	0.001
CD53	membrane	132 ± 94	209 ± 195	0.03	0.7	0.002
IGFR	membrane	1001 ± 905	1169 ± 984	NS	0.7	0.002
CD55	membrane	3698 ± 1380	4686 ± 1966	0.01	0.7	0.002
CD81	membrane	10791 ± 12282	9413 ± 8913	NS	0.7	0.002
HER2/neu	membrane	1096 ± 825	1297 ± 1177	NS	0.6	0.009
CD59	membrane	25045 ± 19142	28607 ± 17346	NS	0.6	0.009
CD38	membrane	994 ± 1076	932 ± 1055	NS	0.6	0.01
CD44	membrane	1425 ± 1083	1713 ± 1422	NS	0.6	0.01
CD63	membrane	1835 ± 1011	2783 ± 1062	0.003	0.5	0.02
CD200	membrane	550 ± 546	378 ± 348	NS	0.5	NS
Bcl2	cytoplasmatic	553 ± 353	684 ± 414	NS	0.4	NS
CD2	membrane	173 ± 87	277 ± 133	0.005	0.2	NS

Markers that were systematically negative are not listed in the table.

* Results expressed as mean MFI ± one standard deviation (SD).

NS, statistically not significant; MFI, mean fluorescence intensity.

3.2. Morphological and genetic features of purified cell populations

Morphological and genetic analyses performed on highly-purified cell populations confirmed coexistence of meningeal tumor cells and non-meningeal infiltrating inflammatory cells in every meningioma sample (Figure 13). Accordingly, both $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ and $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells displayed the cytogenetic alterations detected in the tumor, e.g. del(22q), in association with morphological features consistent with those of tumor cells (e.g. large nuclei, granular chromatin and thick nuclear membrane); whereas $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells showed abundant cytoplasm, $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ cells corresponded to bare nuclei (Figure 13A). In turn, the two $CD45^{+}$ cell populations systematically lacked on such genetic alterations (Figures 13B and 13C). Morphologically, $SSC^{lo}CD45^{hi}HLA\text{-}DR^{het}CD44^{hi}$ cells displayed a typical appearance of mature lymphocytes, consisting of round small cells with scarce cytoplasm, whereas $SSC^{lo}CD45^{+}HLA\text{-}DR^{hi}CD44^{het}$ cells displayed an irregular nuclei and

more abundant cytoplasm, with a morphological appearance compatible with tissue macrophages (Figures 13B and 13C).

Of note, the overall percentage of $CD45^-$ tumor cells (71%±22%) detected by MFC showed a significant correlation (r^2=0.62; p<0.001) with the percentage of cytogenetically altered tumor cells detected by iFISH (65%±21%) in the same samples.

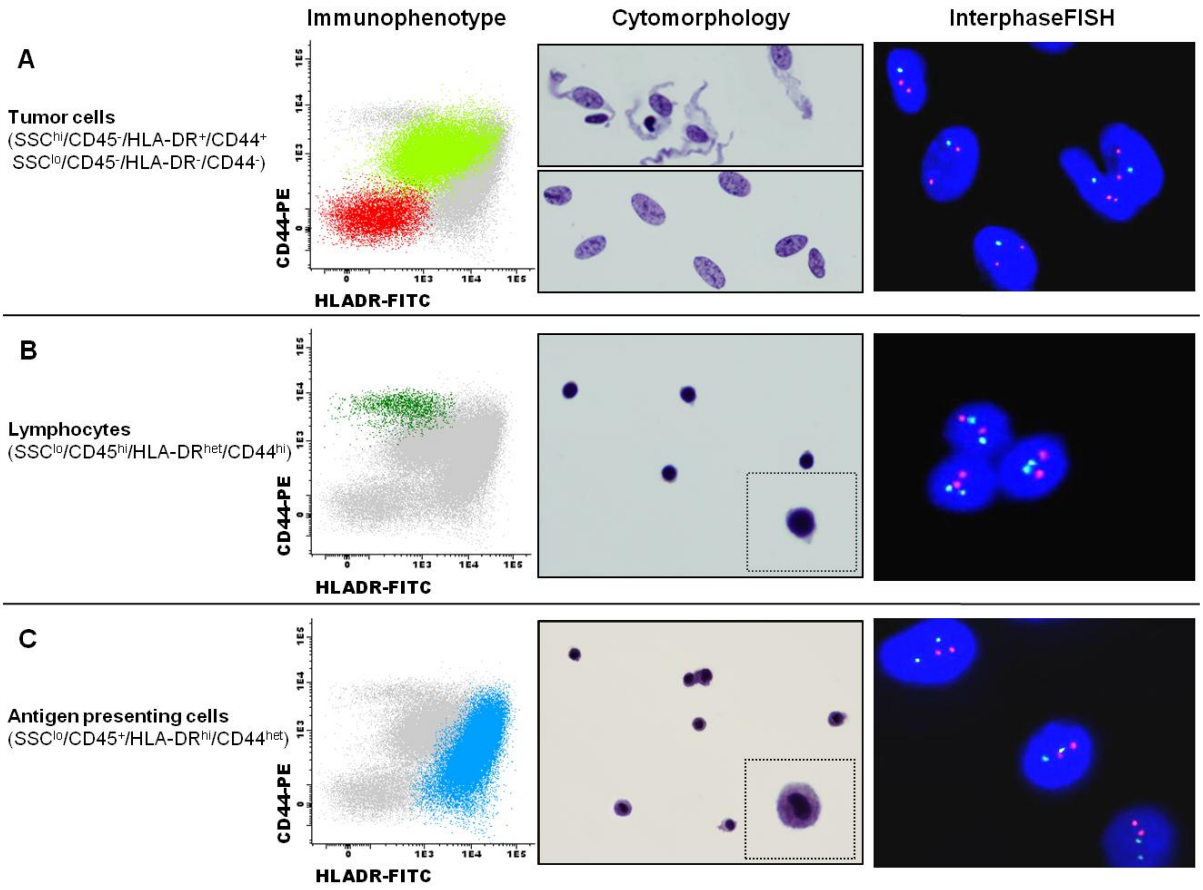

Figure 13. Cytomorphological and genetic characterization of FACS-sorted cell populations present in a representative meningioma tissue sample. Based on multiparameter flow cytometry immunophenotyping, three major populations of cells were systematically identified: tumor cells (panel A), lymphocytes (panel B) and antigen presenting cells (APC, panel C). Among the tumor cell population, two subsets were also found (FSC/$SSC^{hi}CD45^-HLA\text{-}DR^+CD44^+$ light green dots and FSC/$SSC^{lo}CD45^-HLA\text{-}DR^-CD44^-$ red dots - left image, panel A). Lymphocytes systematically showed a FSC/$SSC^{lo}CD45^{hi}HLA\text{-}DR^{het}CD44^{hi}$ phenotype (dark green dots - left image, panel B) while tissue macrophages presented a FSC/$SSC^{lo}CD45^+HLA\text{-}DR^{hi}CD44^{het}$ phenotype (blue dots - left image, panel C). Cytomorphological features of the two subsets of tumor cells, lymphocytes and tissue macrophages as per the Papanicolau stain (original magnification x1000) are shown in the middle image of each panels A, B and C, respectively; the images on the right of the panels show iFISH analysis of chromosomes 9p34 and 22q11.2 (9p34/22q11.2 red/green dual color probe), with two copies of chromosomes 9 and 22 in all lymphocytes and tissue macrophages, while tumor cells from the same meningioma sample displayed del(22q), as reflected by a single green spot/nuclei. No differences were observed between the iFISH probes in the two subsets of tumor cells but cytomorphologic analysis showed a disrupted cytoplasm with bare nuclei in one of them (middle image, panel A).

3.3. Relationship between overall mRNA and protein expression profiles

When considering the overall cellularity of tumor samples, a significant direct correlation was found between the mRNA and the protein expression levels for the CD13 ectoenzyme (r^2=0.9; p<0.001), the CD58 (r^2=0.7; p=0.004) and CD99 (r^2=0.8; P=0.003) cell adhesion molecules, and HLA-DR (r^2=0.7; p=0.01). Conversely, an inverse correlation (r^2=-0.7; p=0.006) between the mRNA and the protein levels was observed for the HER2/neu growth factor receptor (Table 8). No significant correlation was found between mRNA and protein levels for the other 17 proteins analyzed. Noteworthy, a similar pattern and degree of correlation was observed for the studied proteins, when we considered the protein expression levels specifically found for meningeal tumor cells (Table 8). Despite this, a significant direct correlation was found between the mRNA levels of the EGFR and HER2/neu growth factor receptors and the percentage of tumor cells in the sample (r^2>0.5; p<0.05; data not shown) while an inverse correlation was found for HLA-DR, HLA-I, bcl2, CD45, CD14, CD16, CD53 and CD99 (r^2≤-0.5; p<0.05; data not shown).

Table 8. Correlation between the mRNA levels and the mean amount of protein expressed per cell for 22 markers analyzed in 13 meningiomas (protein levels were evaluated both for the overall cellularity of the sample and specifically also for the meningioma tumor cells).

MFC protein	Correlation coefficient			
	Overall cellularity		Meningeal-tumor cells	
	r^2	P-value	r^2	P-value
CD45	0.5	NS	0.2	NS
HLA-I	0.01	NS	-0.07	NS
CD81	0.1	NS	0.3	NS
CD9	-0.3	NS	-0.1	NS
CD13	0.9	0.0001	0.8	0.001
CD38	0.04	NS	-0.1	NS
EGFR	-0.05	NS	0.2	NS
CD2	0.2	NS	-0.2	NS
CD99	0.8	0.003	0.7	0.009
CD16	0.1	NS	-0.5	NS
CD14	0.4	NS	0.3	NS
HLA-DR	0.7	0.01	0.7	0.01
CD44	0.4	NS	0.4	NS
CD55	-0.5	NS	-0.4	NS
CD53	0.5	NS	0.5	NS
CD63	-0.3	NS	-0.1	NS
IGFR	0.1	NS	0.2	NS
HER2Neu	-0.7	0.006	-0.6	0.02
CD59	-0.1	NS	0.2	NS
CD58	0.7	0.004	0.7	0.01
Bcl2	0.5	NS	0.6	0.05
CD69	0.5	NS	0.1	NS

NS, statistically not significant; for mRNA levels the mean value obtained for all probes in the array specific for the corresponding protein mRNA, was used.

4. Discussion

Meningiomas are heterogeneous tumors which consist of both neoplastic cells and other infiltrating non-immune and immune cells (e.g. macrophages/microglial cells and lymphocytes); the latter cells have been suggested to play an important role in modulating the growth and immunogenicity of meningiomas [272, 274, 276, 348]. Although each of these cellular components displays a uniquely different gene expression mRNA and protein profile, to the best of our knowledge, no study has been reported so far, in which the most represented cell populations have been systematically identified and characterized in meningioma samples.

Overall, our results confirm the heterogeneous cellular composition of meningiomas which, together with a major fraction of neoplastic cells, systematically showed variable infiltration by tissue macrophages and to a less extent, also T, NK and a few B cells. Simultaneous identification of the different cell populations was optimally achieved based on differential MFC patterns of expression of CD45, HLA-DR and CD44 by nucleated cells ($DRAQ5^{hi}$). Based on CD45, two major groups of cells were identified: $CD45^{-}$ neoplastic cells and $CD45^{+}$ infiltrating immune cells. Among the latter cells, a majority showed an $HLA\text{-}DR^{+}CD14^{+}CD68^{+}CD16^{-/+}CD33^{-/+}$ phenotype consistent with a monocytic/macrophage lineage origin, as also supported by their high phagocytic and endocytic ability and their morphological appearance. Altogether, these findings support previous observations which reported infiltration by macrophages in meningiomas [272, 274, 276, 348]. However, although multiple reports describe infiltration of different tumor types by tissue macrophages [391], little is known about their phenotype and functional properties in meningiomas. As could be expected, tumor macrophages expressed HLA-I and the CD55 and CD59 complement regulatory proteins, in association with partial positivity for the CD13 (aminopeptidase N) [392] and CD38 ectoenzymes [393], bcl2 [394], activation induced CD69 [395], multiple adhesion molecules (e.g. CD2 [396], CD44 [397], CD58 [398] and CD99 [399]) and several tetraspanins, involved in the organization of microdomains essential for the regulation of signaling pathways central to macrophage activation [400]. These infiltrating immune cells may play an important role in tumor immunology, through complex relationships with tumor cells and other cells in the tumor microenvironment [391]. Currently, tissue macrophages are grouped into M1 and M2 cells, according to the pattern of cytokines they secrete [391]. Several studies suggest that tumor infiltrating macrophages (e.g. in gliomas) exhibit features of M2-like macrophages [294, 401, 402], promoting tumor progression [403]. However, M1-like macrophages have also been detected in some tumors where they are associated with a better prognosis [404, 405]. Further

studies are required to determine the M1/M2-like nature of tissue macrophages in meningiomas and their impact on the disease.

Other less represented $CD45^+$ immune cells ($CD45^{hi}$) corresponded to cytotoxic T CD8+ and NK cells. These results confirm previous findings which suggest that such tumor infiltrating lymphocytes (TIL) in meningioma [272] and also other tumors [406] mainly consist of $CD8^+$ cytotoxic T cells; nevertheless, these cells are frequently unable to control tumor growth and progression [406]. Whether this is due to a specific functional defect of such cytotoxic cells, associated or not to an inhibitory effect induced by standing Treg cells, remains to be elucidated. In this regard, our results show the absence of $CD4^+CD25^{hi}CD127^{lo}$ Tregs in the majority of the meningiomas analyzed, supporting the lack of local immune tolerance induced by Tregs. Recent studies show that the presence of Tregs in various cancer types correlates with a poor prognosis [407]. In line with these observations, Jacobs et al. [327] also reported the virtual absence of Tregs in meningiomas, compared to other malignant tumors such as gliomas. Furthermore, our results also show that tumor infiltrating T cells co-express CD28, a molecule which is critical for providing co-stimulatory signals required for T cell activation. This, together with the observed expression of antigen-presenting molecules (both HLA class I and class II) by tumor cells, point to a potential role of inflammatory infiltrates of meningiomas in controlling tumor growth.

Two distinct groups of $CD45^-$ neoplastic cells were found by MFC. However, sorting of the two populations showed that despite they both carried the same cytogenetic markers, only one corresponded to live cells with a $SSC^{hi}CD45^-$HLA-DR^+CD44^+ phenotype and typical morphological characteristics of meningioma cells. The other $SSC^{lo}CD45^-$HLA-DR^-CD44^- subset consisted of bare nuclei, probably generated during sample preparation by the mechanical disaggregation and/or the freezing procedures. This contributes to explain absence of expression of virtually all proteins analyzed in this latter population. Therefore, a question remains about whether MFC immunophenotyping provides a reliable tool to assess protein expression in frozen meningioma samples. In this regard, it should be emphasized that frequently, fresh tumor samples are not readily available for routine MFC immunophenotyping. Therefore, a major goal of our study was to determine the impact of freezing on the pattern of protein expression by meningioma cells. Overall, our results showed a significant correlation for most proteins analyzed in fresh *vs.* freshly-frozen tumor samples; despite this, higher protein levels were frequently found after freezing. Such differences could be due to a better preservation of PEP in frozen samples; however, they may also reflect simultaneous detection of proteins at the membrane and cytoplasmic cell compartments, due to permeabilization of cells induced by the freezing process. Further studies are required to confirm these hypotheses;

in the meanwhile, our results support usage of frozen instead of fresh tumor tissues for more accurate MFC evaluation of overall PEP in meningiomas.

Another major goal of our study was to determine the relationship between gene expression profiles at the mRNA *vs.* the protein level. Interestingly, CD13, CD58, CD99 and HLA-DR were the only proteins for which a significant correlation between protein and mRNA levels was observed. For the other proteins analyzed, either no correlation or an even negative significant correlation (e.g. HER2/neu) was found. Altogether, these results indicate that mRNA levels frequently do not reflect the amount of protein expressed by individual tumor cells [408]. Such discrepancies could be expected since the level of expression of membrane proteins does not only depend on their synthesis, but also on other factors such as post-translational modifications, a balance between protein synthesis, degradation and secretion and/or the mobilization of previously stored proteins, as extensively described in previous studies comparing mRNA and protein levels [408-410]. Of note, the integrity of the extracted RNA determined by microfluidic electrophoresis was confirmed in our study by the high quality RNA obtained, which would rule out potential RNA degradation during tumor disaggregation procedures. Similarly, mechanical disaggregation of the tissue required for MFC immunophenotyping, could also had an impact on the levels of expression of proteins in individual cells. If this holds true, then the negative correlation observed between protein and mRNA levels for some markers, i.e. between HER2/neu mRNA and protein levels, could not be clearly explained. In fact, such inverse correlation potentially reflects specific internalization and/or cleavage of HER2/neu due to recycling of the receptor between the plasma membrane and the endosomal compartments, in addition to protease-mediated cell surface cleavage in activated cells [411]. In line with this hypothesis, previously reported studies indicate that mechanical disaggregation is a better technique than enzymatic methods for protein evaluation in individual cells from solid tumor samples by MFC [412, 413]. Since mRNA studies are performed with the whole tumor sample, including both neoplastic and infiltrating immune cells, we wondered whether the cellular heterogeneity of the tumor could also have an impact on the gene expression profiles. Interestingly, a positive correlation was found between the mRNA levels of proteins specifically expressed by tumor cells (e.g. EGFR and HER2/neu) and the percentage of neoplastic cells in the tumor sample, while markers highly (or exclusively) expressed by the infiltrating inflammatory cells (e.g. HLA-DR, HLA-I, bcl2, CD45, CD14, CD16 and CD53) were inversely correlated with the tumor cell contents of the sample. Altogether, these results indicate that microarray-based mRNA expression profiles partially reflect the cellular composition of the tumor rather than precise features of cancer cells, while

evaluation of gene expression at the protein level by MFC would more closely reflect the phenotypic profile of neoplastic cells.

Detailed MFC analysis of the immunophenotypic characteristics of tumor cells in meningiomas showed constant expression of several adhesion-associated molecules, such as the CD13 ectoenzyme [414], CD44 [92] and CD9, CD63 and CD81 tetraspanins [415-418], which may play an important role in the regulation of tumor cell motility, proliferation and intracellular signaling. Interestingly, CD13 expression in meningiomas has been previously reported to be inversely associated with a more indolent disease behavior [419], in line with the high levels of CD13 detected in our cohort, mainly composed of WHO grade I meningiomas. Additionally, expression of CD44, which has been also previously described in meningiomas [375, 420], emerges as a potentially relevant molecule in these tumors since signaling through CD44 inhibits merlin, a protein coded in chromosome 22 whose expression is frequently lost in meningiomas [421, 422]. Conversely, to the best of our knowledge, this is the first study in which expression of tetraspanins is analyzed in meningioma cells, showing a unique pattern of CD9, CD63 and CD81 expression, associated with variable levels of CD53, in the absence of CD37.

Previous studies have highlighted the relevance of anti-apoptotic proteins [423] and growth factor receptors [424-428] in meningioma cell growth and survival, due to their association with both tumor histopathology and patient outcome [156, 310, 424, 425, 428]. In line with these observations, we found heterogeneous patterns of expression of HER2/neu, IGFR and EGFR in meningiomas, together with variable levels of positivity for the anti-apoptotic bcl-2 protein. Further studies, in which the impact of the patterns of expression of these proteins is investigated, are required to determine their clinical value.

In recent years, tumor cell lysis through complement-activated proteins has been identified as a relevant cytotoxic mechanism that could be exploited for novel cancer-targeted therapies. Interestingly, Shinoura et al. [429] reported low mRNA expression of the CD55 and CD59 complement regulatory molecules in meningiomas, which would support targeting tumor cells by such therapies. However, our results show high levels of both proteins on the tumor cell membrane, which would potentially protect them from bystander injury when complement is activated [430]. Interestingly, expression of HLA-I was also systematically detected in meningioma cells, which could favor the control of tumor growth since HLA-I is involved in the presentation of self tumor antigens during immune responses by cytotoxic cells against intracellular proteins [431].

Noteworthy, meningioma cells from most tumors shared expression of two molecules characteristic of tissue macrophages: HLA-DR and CD14. Despite HLA class II antigen

expression is generally restricted to professional antigen-presenting cells (APC) and thymic epithelial cells, HLA class II^+ tumor cells have been also recurrently found in breast and colorectal carcinomas, in association with a better patient outcome [432, 433]. This could be related to the fact that HLA class II^+ tumor cells may facilitate induction of anti-tumoral T-cell responses by $CD4^+$ Th1 lymphocytes, indicating that expression of determinants of the immune response by tumor cells may influence tumor progression and patient outcome [432, 434]. Interestingly, in the present study we not only demonstrate that tumor cells co-express HLA-II (e.g. HLA-DR) but they also display a significant endocytic activity, a function typically required by distinct cell types, including APC, to up-regulate expression of HLA-II [435]. This, together with the expression of the TLR-associated CD14 molecule suggests that neoplastic cells from meningiomas could play a critical role in priming and controlling local inflammatory and T-cell immune responses. In contrast to glial cells, meningeal cells have not been ontogenetically linked with the monocyte/macrophage/dendritic cell lineages. However, expression of HLA-DR by meningioma cells has been previously reported by others [272, 384]. Likewise, expression of CD14 has also been found in cell types other than the monocytic/macrophage lineage [436-439], including meningeal cells [440, 441]. Based on similar observations, Shabo et al., [442] have suggested that such mixed phenotypes could result from heterotypic cell fusion between primary cancer cells and tumor-associated macrophages. However, it should be noted that absence of DNA aneuploidy in most meningioma cells, as assessed by the DRAQ5 and other DNA staining [48], would rule out such possibility. Further studies are necessary to better understand the role of HLA class II^+ tumor cells in meningiomas.

In summary, here we propose a new 4-colour MFC-based strategy for the evaluation of the cellular composition of meningiomas. Overall, our results show systematic presence of inflammatory and other immune cells coexisting with variable numbers of neoplastic cells, such infiltrating inflammatory cells mainly consisting of tissue macrophages and to a lesser extent, cytotoxic $TCD8^+$ and NK cells. Further analysis of the PEP in fresh *vs.* frozen samples showed identical profiles, although the freezing process may have a moderate impact on preserving the levels of expression of individual proteins. At last, here we show that MFC immunophenotyping provides a more reliable way of assessing gene expression by tumor cells at the protein/functional level, compared to mRNA levels assessed by microarrays.

Chapter V

Association between inflammatory infiltrates and isolated monosomy 22/del(22q) in meningiomas

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(Note: additional results from those published in the journal were included in this chapter)

1. Abstract

Meningiomas contain highly variable levels of infiltrating tissue macrophages (TiMa) and other immune cells. In this study we investigated the potential association between the number and immunophenotype of inflammatory and other immune cells infiltrating the tumor as evaluated by multiparameter flow cytometry, and the clinico-biological, cytogenetic and gene expression profile (GEP) of 75 meningioma patients. Overall, our results showed a close association between the amount and cellular composition of the inflammatory and other immune cell infiltrates and the cytogenetic profile of the tumors. Notably, tumors with isolated monosomy 22/del(22q) showed greater numbers of TiMa, NK cells and (recently)-activated $CD69^+$ lymphocytes *vs.* meningiomas with diploid and complex karyotypes. In addition, in the former cytogenetic subgroup of meningiomas, tumor-infiltrating TiMa also showed a more activated and functionally mature phenotype, as reflected by a greater fraction of $CD69^+$, $CD63^+$, $CD16^+$ and $CD33^+$ cells. GEP at the mRNA level showed a unique profile among meningiomas with an isolated monosomy 22/del(22q) *vs.* all other cases, which consisted of increased expression of genes involved in inflammatory/immune response, associated with an M1 TiMa phenotype. Altogether, these results suggest that loss of expression of specific genes coded in chromosome 22 (e.g. *MIF*) is closely associated with an increased homing and potentially also anti-tumoral effect of TiMa, which could contribute to explain the better outcome of this specific good-prognosis cytogenetic subgroup of meningiomas.

2. Introduction

Meningiomas are usually considered to be benign central nervous system tumors, on both histopathological [6] and clinical [3] grounds. Despite this, a significant fraction of all meningiomas will eventually relapse with a negative impact in patient outcome [3]. In recent years, tumor cytogenetics has emerged as the most relevant prognostic factor, together with tumor histopathology/grade and patient age and sex [79, 443]. Whereas cases displaying complex karyotypes, particularly monosomy 14 in association with del(1p), display a dismal outcome [60], tumors with isolated monosomy 22/del(22q) show a particularly good prognosis, the molecular basis of such clinical behavior remaining to be fully understood [60, 73, 79].

Although tumor histopathology and tumor behavior are, at least in part, related to tumor cytogenetics [3, 224], they might also be influenced by specific changes in the tumor microenvironment [276, 444]. In this regard, we have recently reported the existence of variable levels of infiltration of meningiomas by inflammatory and other immune cells [445]. $CD45^+$ inflammatory cells that infiltrated meningiomas mainly included tissue macrophages (TiMa) with an $HLA\text{-}DR^+CD14^+CD45^+CD68^+CD16^{-/+}CD33^{-/+}$ phenotype and a high phagocytic/endocytic activity, together with a smaller population of cytotoxic lymphocytes, mostly $CD8^+$ T cells and NK-cells [445]. Previous studies in other tumor types such as melanoma and colorectal cancer [446, 447], have shown that infiltration by immune cells is associated with specific features of the disease, including a better outcome. In meningiomas, previous reports indicate that the type and level of infiltrating inflammatory cells are both associated with the histopathological features of the tumor [272, 276, 445]. However, so far no study has investigated the potential association between tumor infiltrates of inflammatory and other immune cells, and other features of the disease, including cytogenetics.

In this study, we investigated the association between the cellular composition and protein expression profiles of meningiomas as analyzed by multiparameter flow cytometry, and the clinico-biological, genetic and mRNA gene expression profiling features of the disease. Our results indicate that the presence of inflammatory infiltrates of antigen presenting cells (TiMa) and lymphocytes is clearly associated with tumors displaying isolated monosomy 22/del(22q), which could contribute to explain the better outcome of this specific cytogenetic subgroup of meningiomas.

3. Results

3.1. Inflammatory infiltrates in meningioma samples and its association with disease features

As previously described [445], all meningioma samples showed infiltration by inflammatory and other immune cells by flow cytometry, although their percentage was highly variable among the distinct tumors. In order to investigate the potential association between the amount of the inflammatory infiltrate by flow cytometry and other features of the disease, patients were divided into cases with low (<23%) and high (≥23%) percentage of the most represented inflammatory cells ($CD14^+HLA\text{-}DR^+CD45^+$ TiMa) in the tumor infiltrates, based on ROC curve analysis - area under the curve (AUC) of 90% ($p<0.001$) for the selected cutoff (23% of TiMa) -. Immunohistochemical expression of CD68 was detected in the cytoplasm of morphologically heterogeneous mononuclear cells scattered within the tumor tissue, as single cells or groups of cells, only occasionally localized in perivascular areas (Figure 14). On the basis of their immunophenotype, morphology and localization, these cells were identified as mainly corresponding to macrophages infiltrating the tumor.

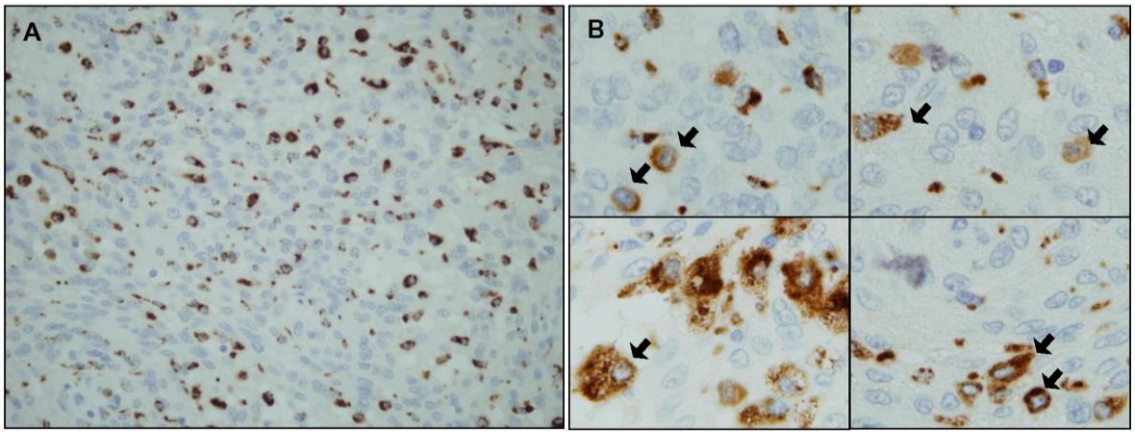

Figure 14. Immunohistochemical staining of meningioma tissues with the anti-CD68 antibody. CD68-positive cells detected within the tumor parenchyma showed reactivity in their cytoplasm and a mononuclear cell appearance, compatible with macrophages infiltrating the tumor. An overview of the whole tissue from a representative case (original magnification, x400) (panel A) and a higher amplification of areas containing CD68-positive cells (original magnification, x1000) (panel B), are displayed.

Comparison of patients with low (<23% TiMa) *vs.* high (≥23% TiMa) levels of tumor infiltration by inflammatory cells (Table 9) showed a clearly different distribution of tumors according to their iFISH cytogenetic profiles ($p=0.001$); conversely, both groups of patients showed a similar distribution according to age, sex, tumor localization, histopathological subtypes and WHO tumor grade, together with a similar degree of brain edema and frequency

of relapses (p>0.05). From the cytogenetic point of view, a highly significant association was found between meningiomas carrying isolated monosomy 22/del(22q) alone and high levels of infiltration by TiMa, most tumors carrying isolated monosomy 22/del(22q) (14/17 cases, 82%) showing infiltration by ≥23% TiMa (Table 9).

Of note, whereas cases with either a diploid karyotype or isolated monosomy 22/del(22q) tumors showed a longer RFS than meningioma patients carrying complex karyotypes (p=0.01), the level of tumor infiltration by TiMa on itself did not show a significant impact on patient outcome (p>0.05).

Table 9. Clinical, biological and cytogenetic characteristics of meningioma patients with high (≥23%) *vs.* low (<23%) degree of tumor infiltration by tissue macrophages (TiMa; n=51).

		Total cases (n=51)	**% of TiMa <23 (n=29)**	**% of TiMa ≥23 (n=22)**	***P-value***
Age (years)		58 ± 13	59 ± 14	56 ± 12	NS
Gender	Female	36 (71%)	19 (66%)	17 (77%)	NS
	Male	15 (29%)	10 (44%)	5 (23%)	
Tumor localization	Convexity	17 (33%)	7 (24%)	10 (45%)	NS
	Cranial base	14 (27%)	11 (38%)	3 (14%)	
	Convexity/Parasagittal	8 (16%)	4 (13%)	4 (18%)	
	Parasagittal	6 (12%)	3 (10%)	3 (14%)	
	Tentorial	2 (4%)	2 (7%)	0 (0%)	
	Intraosseous	1 (2%)	1 (4%)	0 (0%)	
	Spinal	3 (6%)	1 (4%)	2 (9%)	
Tumor grade	Grade I	46 (90%)	25 (86%)	21 (95%)	NS
	Grade II	3 (6%)	2 (7%)	1 (5%)	
	Grade III	2 (4%)	2 (7%)	0 (0%)	
Tumor histopathology	Meningothelial	14 (27%)	9 (31%)	5 (23%)	NS
	Transitional	16 (31%)	10 (35%)	6 (27%)	
	Psammomatous	7 (14%)	3 (10%)	4 (18%)	
	Fibroblastic	7 (14%)	1 (3%)	6 (27%)	
	Other *	7 (14%)	6 (21%)	1 (5%)	
iFISH karyotype	Diploid	17 (33%)	14 (48%)	3 (14%)	0.001
	Monosomy 22/del(22q)	17 (33%)	3 (10%)	14 (63%)	
	Complex	16 (32%)	11 (38%)	5 (23%)	
	del(1p)	1 (2%)	1 (4%)	0 (0%)	
Edema	No/light	30 (59%)	14 (48%)	16 (73%)	NS
	Moderate/severe	21 (41%)	15 (52%)	6 (27%)	
Relapses	Yes	4 (8%)	1 (4%)	3 (14%)	NS
	No	47 (92%)	28 (96%)	19 (86%)	

NS, statistically no significant differences observed (*p*>0.05).
* Includes one angiomatous, one secretory, one rhabdoid, one papillary and three atypical meningioma cases.

3.2. The immunophenotypic profile of inflammatory cells in tumor infiltrates varies according to tumor cytogenetics

A more detailed analysis of the immunophenotypic features of TiMa from meningioma samples with distinct karyotypes showed that cases with isolated monosomy 22/del(22q) not only displayed significant increased levels of infiltration by TiMa *vs.* meningiomas with both diploid ($p<0.001$, Figure 15B) and complex karyotypes ($p=0.02$, Figure 15B), but they also showed a distinct immunophenotypic profile for such TiMa (Figure 16). Accordingly, TiMa from meningiomas with isolated monosomy 22/del(22q) showed increased expression levels of several activation-associated markers with higher percentages of $CD69^+$ ($p\leq0.009$ *vs.* diploid and complex tumors; Figure 16A) and $CD63^+$ TiMa ($p=0.006$ *vs.* diploid cases; Figure 16B). In addition, TiMa from meningiomas with isolated monosomy 22/del(22q) also displayed a higher percentage of $CD16^+$ cells *vs.* tumors with complex karyotypes ($p=0.004$; Figure 16C); despite not statistically significant, higher percentages of $CD33^+$ cells were also associated with -22/22q$^-$ cases (Figure 16D). In turn, they showed intermediate levels of expression of both the CD44 and CD9 adhesion molecules, between those of diploid and complex karyotype tumors ($p<0.05$; Figures 16E and 16F).

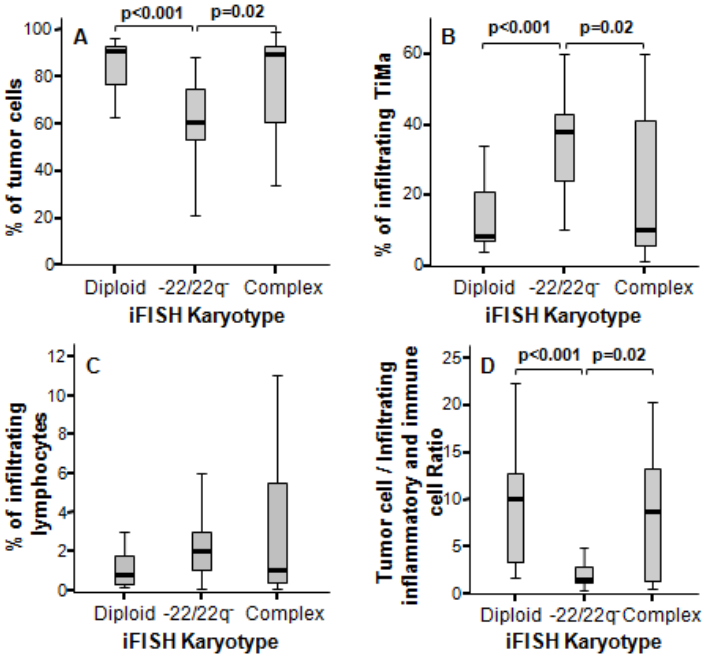

Figure 15. Distribution of tumor cells, inflammatory and other immune cells in meningioma samples classified according to the cytogenetic profile of the tumor. Percentage of tumor cells (panel A), tissue macrophages (TiMa) (panel B) and total lymphocytes infiltrating meningioma samples (panel C), grouped according to the cytogenetic iFISH profile of the tumor, are shown, as also the ratio between the number of tumor cells and all other infiltrating cells (panel D). Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively.

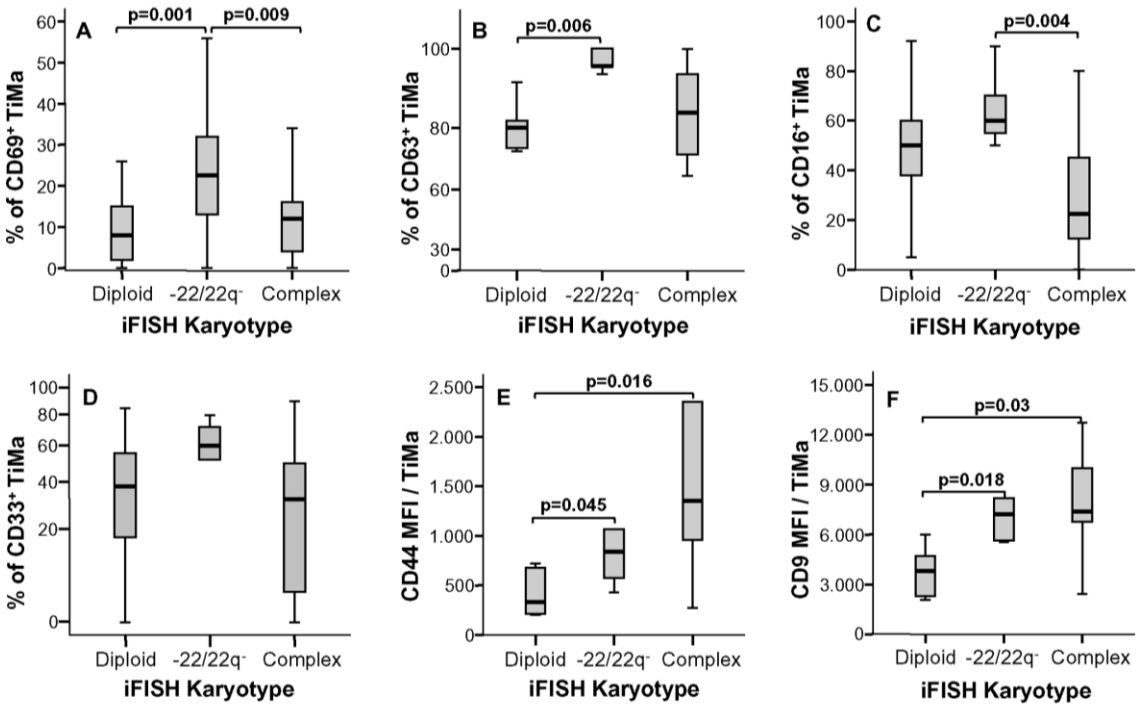

Figure 16. Immunophenotype of tissue macrophages (TiMa) infiltrating meningioma samples, according to the cytogenetic profile of tumor cells. The percentage of TiMa expressing CD69 (panel A), CD63 (panel B), CD16 (panel C) and CD33 (panel D) are shown together with the mean amount of CD44 (panel E) and CD9 (panel F) expressed per TiMa infiltrating meningioma samples, according to the iFISH profile of tumor cells. MFI, mean fluorescence intensity (arbitrary fluorescence units) per cell. Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively.

Conversely, no significant differences (p>0.05) were found regarding the amount of lymphocytes infiltrating the tumor (Figure 15C), neither their major subsets (Figure 17), except for higher numbers of NK-cells in cases with isolated monosomy 22/del(22q) *vs.* diploid tumors (p=0.03; Figure 17E). Interestingly, this was also associated with an increased percentage of CD69^{+} lymphocytes among meningiomas with isolated monosomy 22/del(22q) *vs.* both cases with diploid and complex iFISH karyotypes (p<0.05; Figure 17D).

In line with the above observations, unsupervised hierarchical clustering analysis, based on the number and immunophenotypic features of the inflammatory tumor infiltrates of each meningioma, showed two major clusters of tumors (Figure 18A): one group (group A) included the great majority of patients (13/17, 76%) with meningiomas carrying isolated monosomy 22/del(22q), whereas the other group (group B) comprised most patients with a diploid (14/17, 82%) or complex karyotype (10/16, 63%). As expected, group A cases were characterized by both higher levels of infiltration by TiMa and lymphocytes, and greater

percentages of CD69$^+$ activated TiMa and lymphocytes. Additionally, principal component analysis based on the percentages of lymphocytes and TiMa infiltrating meningioma samples and their immunophenotypic features, also identified a homogeneous group of samples (n=13/51) which included almost only cases with isolated monosomy 22/del(22q) (11/13, 85%; Figure 18B). Of note, from the three iFISH cytogenetic subgroups of meningiomas, the complex karyotype subgroup showed more variable levels of infiltration by TiMa (Figure 15B) and lymphocytes (Figures 15C and 17). Thus, 5/16 cases with a complex karyotype showed high levels of TiMa infiltrates (Table 9), and 6/16 were misallocated in group A in the cluster analyses (Figure 18A).

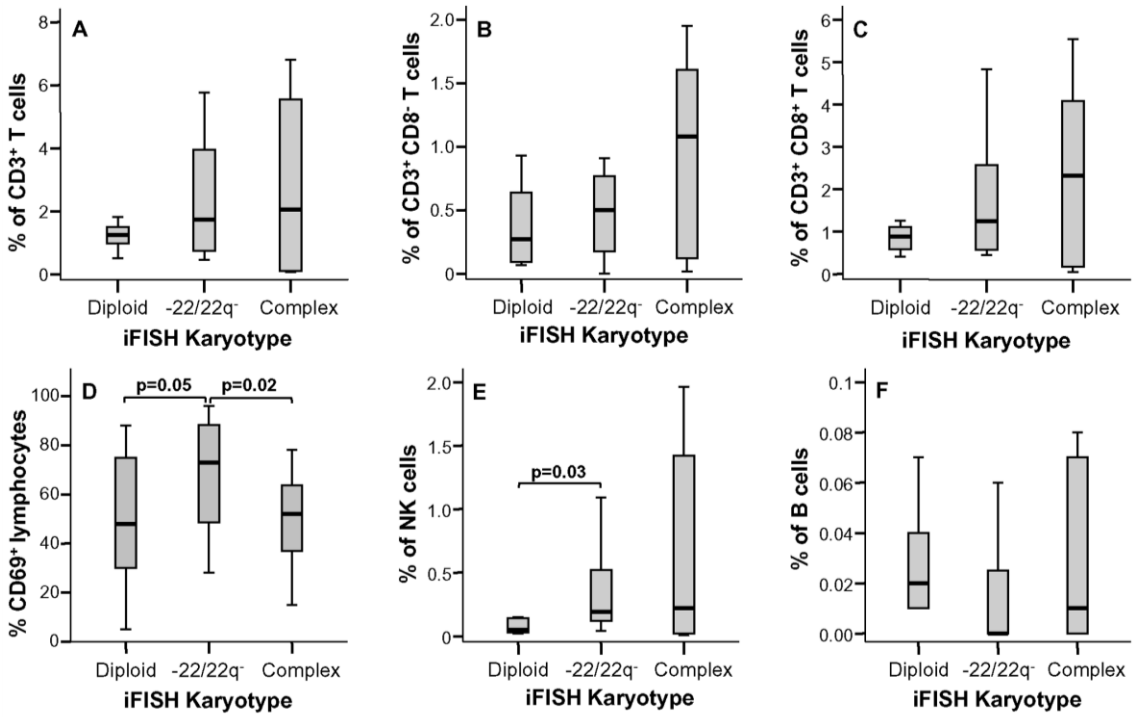

Figure 17. Distribution of the major lymphocyte subsets and activated CD69$^+$ lymphocytes in inflammatory infiltrates of meningiomas classified according to the cytogenetic profile of tumor cells. The percentage of total CD3$^+$ T cells (panel A), CD3$^+$CD8$^-$ T cells (panel B), CD3$^+$CD8$^+$ T cells (panel C), activated CD69$^+$ lymphocytes (panel D), CD3$^-$CD19$^-$CD56$^+$ NK cells (panel E) and CD3$^-$CD19$^+$ B cells (panel F) are shown. Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively.

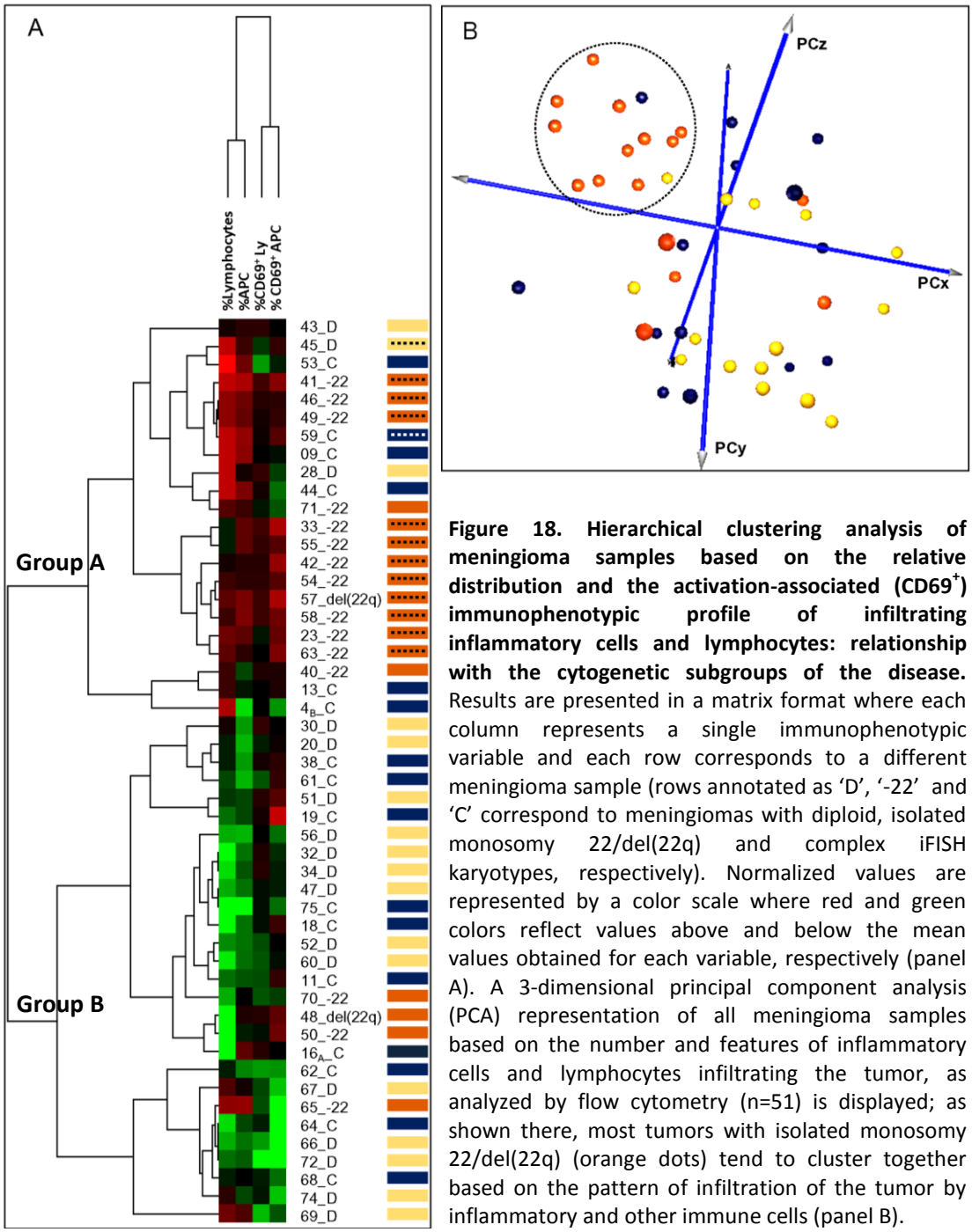

Figure 18. Hierarchical clustering analysis of meningioma samples based on the relative distribution and the activation-associated ($CD69^+$) immunophenotypic profile of infiltrating inflammatory cells and lymphocytes: relationship with the cytogenetic subgroups of the disease. Results are presented in a matrix format where each column represents a single immunophenotypic variable and each row corresponds to a different meningioma sample (rows annotated as 'D', '-22' and 'C' correspond to meningiomas with diploid, isolated monosomy 22/del(22q) and complex iFISH karyotypes, respectively). Normalized values are represented by a color scale where red and green colors reflect values above and below the mean values obtained for each variable, respectively (panel A). A 3-dimensional principal component analysis (PCA) representation of all meningioma samples based on the number and features of inflammatory cells and lymphocytes infiltrating the tumor, as analyzed by flow cytometry (n=51) is displayed; as shown there, most tumors with isolated monosomy 22/del(22q) (orange dots) tend to cluster together based on the pattern of infiltration of the tumor by inflammatory and other immune cells (panel B).

3.3. Association between CD206-positive TiMa, Treg infiltration and tumor cytogenetics

In order to better understand the phenotype and functional role of the meningioma-infiltrating TiMa, as well as the mechanisms responsible for the heterogeneity found in the complex karyotype subgroup of meningiomas, the CD206 M2-marker was evaluated in a different series of meningiomas. Overall, CD206 expression was detected in 23%±19% of the whole TiMa with mean CD206 MFI of 492±797 arbitrary units. Of note, no significant

association was found between CD206 expression by TiMa and tumor cytogenetics, as defined by the three iFISH subgroups - 14/33 diploid, 5/33 monosomy 22/del(22q), and 14/33 complex karyotype tumors - (Supplementary Table 1). In contrast, complex karyotype tumors which showed higher level of infiltration by TiMa (6/14 complex karyotype meningiomas) displayed higher levels of expression of CD206 *vs.* meningiomas with monosomy 22/del(22q) (Table 10), as reflected by significantly greater percentages of $CD206^{+}$ TiMa (39%±20% *vs.* 13%±10%, respectively; p=0.02). In addition, this subgroup of meningiomas carrying a complex karyotype associated with high levels of infiltration by TiMa also showed significantly higher numbers of $CD4^{+}CD25^{hi}CD127^{-/lo}$ Treg cells (289±510x10^{-5} *vs.* 9±10x10^{-5} Tregs, respectively; p=0.01), in association with lower NK cell numbers (9%±9% *vs.* 28±8%, respectively; p=0.02; Table 10), compared with meningiomas carrying isolated monosomy 22/del(22q). Regarding CD163, 2/5 cases showed expression of this marker in 25%±21% of the TiMa infiltrates (MFI 231±233), from which one was a complex tumor with high levels of TiMa infiltration, and the other corresponded to a diploid case with low levels of TiMa infiltration. Figure 19 shows two representative meningiomas with complex karyotypes and high levels of infiltration by TiMa associated with increased expression of CD206, and infiltration by Tregs.

Table 10. Levels of tumor-infiltration by lymphocyte (TIL) subpopulations, with particular focus on regulatory T cells identified as $CD4^{+}CD25^{hi}CD127^{-/lo}$ cells, and infiltrating tissue macrophages (TiMa) with expression of the M2 phenotype-associated marker CD206, in meningiomas with isolated monosomy 22/del(22q) and complex karyotype tumors with high level of TiMa infiltrates, selected from the additional series of 33 meningiomas analyzed for CD206 expression.

	ID	%TIL *	%B cells #	%NK cells #	%TCD8 #	%TCD4 #	%Treg #	No. Treg / 100000 cells	%TiMa *	TiMa CD206 (MFI)	%TiMa $CD206^{+}$ #	%TiMa $CD206^{+}$ *
Complex karyotype (with high TiMa infiltration)	76	20	2.6	10	60	27	0.3	58	40	83	15	6
	77 R	6	1	28	39	33	1.2	52	43	544	30	13
	78	4	1.9	6	65	27	0.4	13	32	1008	60	19
	83 R	12	5.7	4	58	32	0.4	52	42	1142	60	25
	84	44	0.8	4	46	49	3.6	1318	17	571	50	9
	104 &	10	0	4	72.5	23.5	3	242	60	275	20	12
Mean ± SD		16 ± 15	2 ± 2	9 ± 9	57 ± 12	32 ± 9	1.5 ± 1.5	289 ± 510	39 ± 14	604 ± 409	39 ± 20	14 ± 7
Monosomy22 / del(22q)	89	1	2.6	35	24	38	1	6	20	143	10	2
	90	0.8	1.3	32	43	23	0.4	2	13	111	10	1
	94	5	0.8	29	41	30	0.9	25	50	54	5	3
	96	2	0.7	27	54	18	0.1	2	54	524	30	16
	97	0.5	3.2	15	42	40	2.7	10	30	167	10	3
Mean ± SD		2 ± 2	2 ± 1	28 ± 8	41 ± 11	30 ± 10	1 ± 1	9 ± 10	33 ± 18	200 ± 186	13 ± 10	5 ± 6
p-value		*0.01*	*0.02*					*0.01*		*0.07*	*0.02*	*0.04*

MFI, mean fluorescence intensity; TIL, tumor-infiltrating lymphocytes.
Results are expressed as the percentage of positive cells for: (*) the whole tumor, or (#) a specific population (TIL or TiMa);
& Case also stained for CD163; R Recurrent tumor.

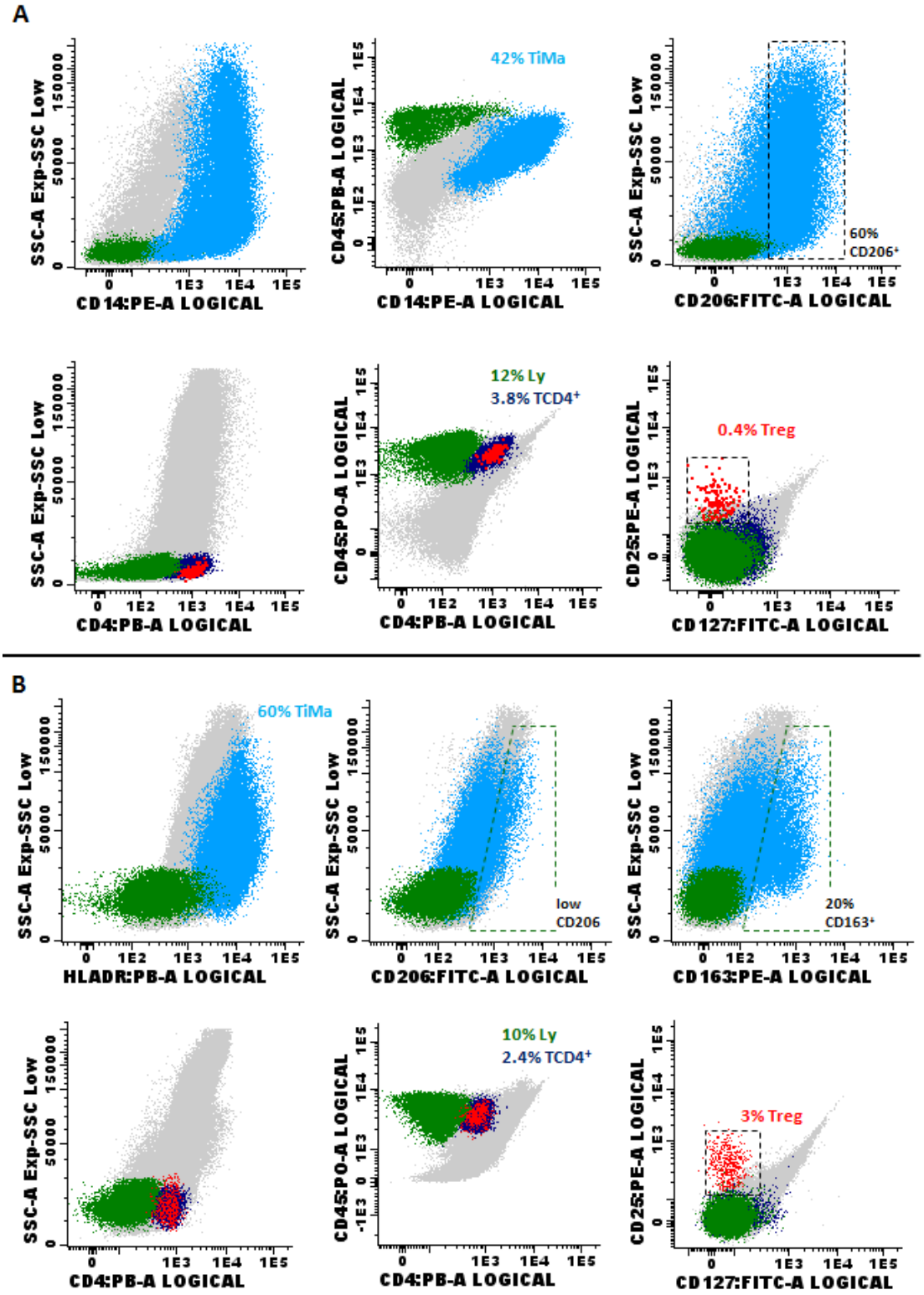

Figure 19. Illustrating example of two cases from the additional series of 33 samples stained for CD206 and T regulatory cells (Tregs). Two complex karyotype cases with high infiltration levels by $CD45^{+}$HLA-$DR^{+}CD14^{+}$ tissue macrophages (TiMa) are shown. **(A)** In the first case, 60% of TiMa are positive for the CD206 M2 phenotype-associated marker, along with 0.4% of lymphocytes with a $CD4^{+}CD25^{hi}CD127^{-/lo}$ phenotype (Tregs). **(B)** In the second case, despite low expression levels of CD206**,** TiMa show a higher expression of the CD163 M2-associated marker, in addition to 3% $CD4^{+}CD25^{high}CD127^{-/low}$ Treg cells among the tumor-infiltrating lymphocyte population.

3.4. Association between the gene expression profile and iFISH karyotype of meningiomas and the pattern of infiltration of the tumor by inflammatory and other immune cells

In order to better understand the molecular mechanisms that could contribute to explain the association observed between the pattern of infiltration of meningioma samples by inflammatory cells and the tumor iFISH karyotype, we further investigated the GEP of 40 meningioma samples using DNA oligonucleotide arrays. Unsupervised hierarchical clustering analysis using those 79 genes, for which the highest variation among tumors with distinct iFISH cytogenetic patterns was observed, showed clear separation among meningioma samples displaying distinct karyotypes, according to their mRNA expression profiles (Figure 20). Analysis of the functional role of these 79 genes associated with the different cytogenetic groups of meningiomas, showed that cases with isolated monosomy 22/del(22q) were specifically characterized by an increased expression of a set of genes which are related to the inflammatory response and to signaling/activation of immune cells. Among other genes, these included the *BCL2*, *C3AR1*, *CD37*, *CLEC7A*, *ELN*, *HLA-DMA*, *HOXC4*, *ITGAM*, *LTBP2*, *MYO1F*, *PIK3CD*, *PLCB1* and *TLR2* genes (Figure 20). Conversely, diploid tumors were mainly characterized by overexpression of a group of genes, (e.g. *ABCB1*, *ADSL*, *CHKB*, *PACSIN2*, *PMM1* and *TCN2* genes) which are mainly involved in small molecule metabolism and cellular biochemistry, including also the *NF2* gene. Finally, tumors with complex karyotypes were characterized by a greater expression of the *ALDOA*, *TRA1*, *NME1*, *NPLOC4* and *TMED9* genes, as well as by decreased levels of the *ALPL*, *COL8A2*, *EFS*, *GSTM1*, *GSTM5*, *KCNMA1*, *KNS2*, *LEPR*, *LPHN2*, *LTBP1*, *MAP3K5*, *PACS2*, *SFRP1*, *TIMP3* and *ZFYVE21* genes, most of such genes being mainly involved in cellular functions related to cell death, cell cycle, cell growth and proliferation, and to cellular assembly.

A more detailed functional analysis of the specific inflammatory pathways involved in meningiomas with isolated monosomy 22/del(22q) (IPA software) showed involvement of inflammatory response genes which are specifically associated with immune responses, cell adhesion, motility and activation and recruitment of antigen presenting cells and/or macrophages (Figure 21). Altered genes included HLA and HLA-associated molecules (*HLA-DMA*, *HLA*-DMB, *HLA-DRA*, *HLA-DRB1*, *HLA-DQA1*, *HLA-DQB* and *CD74*), inflammatory cytokines (*IL16*, *IL1B*, *IL1R1*, *IL10RA*, *IL11RA* and *IL17RA*), complement proteins (*C5*, *C3*, *C3AR1* and *C5AR1*), immunoglobulin Fc (FcIg) receptors (*FCGR1A*, *FCGR2A*, *FCGR3B* and *FCER1G*) and the *CCR1* chemokine receptor, integrins (*ITGAM*, *ITGAX*, *ITGA4* and *ITGB2*) and other adhesion molecules (*VCAM1*, *CD53*, *CD58*, *CD81* and *CD93*), immune co-stimulatory molecules (*CD4*, *CD40* and *CD86*), toll-like receptors (*TLR2*, *TLR5* and *TLR7*) and TLR-associated molecules (*CD14*

and *MYD88*), growth factors and growth factor receptors (*CSF1*, *CSF1R* and *IGF1*), apoptosis-associated proteins (*BCL2* and *BID*), together with phosphoinositide-3-kinases (*PIK3CG* and *PIK3CD*) and other kinases (*PRKCD*, *SYK*, *LYN* and *HCK*), tyrosine phosphatases (*PTPRC* and *PTPN6*), and signaling molecules (*CD69*, *CYBB*, *GAB2*, *HIF1A*, *INPP5D*, *IRF8*, *MSR1*, *SEMA4D*, *TREM2*, *TYROBP* and *WAS*) (Figure 21).

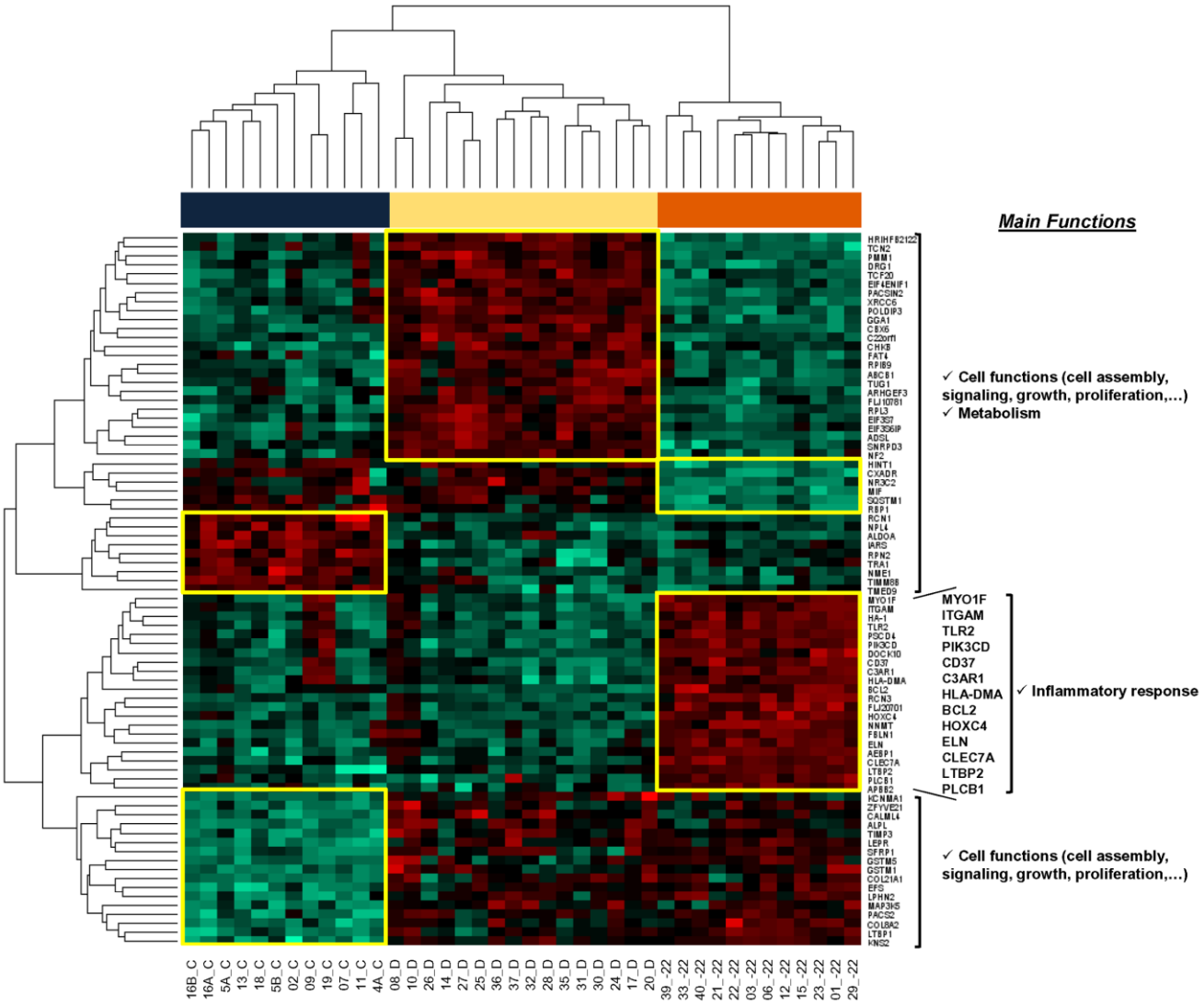

Figure 20. Hierarchical clustering analysis of the GEP of meningioma samples. Results are presented in a GEP matrix format where each row represents a single gene (listed with the corresponding gene symbol) and each column corresponds to a distinct meningioma sample (n=40); those columns identified as 'D' (colored yellow), '-22' (colored orange) and 'C' (dark blue color) correspond to individual meningioma tumors with a diploid, monosomy 22/del(22q) and complex iFISH karyotype, respectively. Normalized values are represented by a color scale where red and green colors indicate values above and below the mean mRNA expression values, respectively. Hierarchical clustering analysis was based on the expression of those 79 genes which showed the highest classification power for the three cytogenetic subgroups of meningiomas. On the right side of the plot, the major common functions of the listed genes, based on the analyses performed with the Ingenuity Pathway software, are indicated. As displayed, genes overexpressed in meningiomas carrying monosomy 22/del(22q) are mainly involved in inflammatory cell functions.

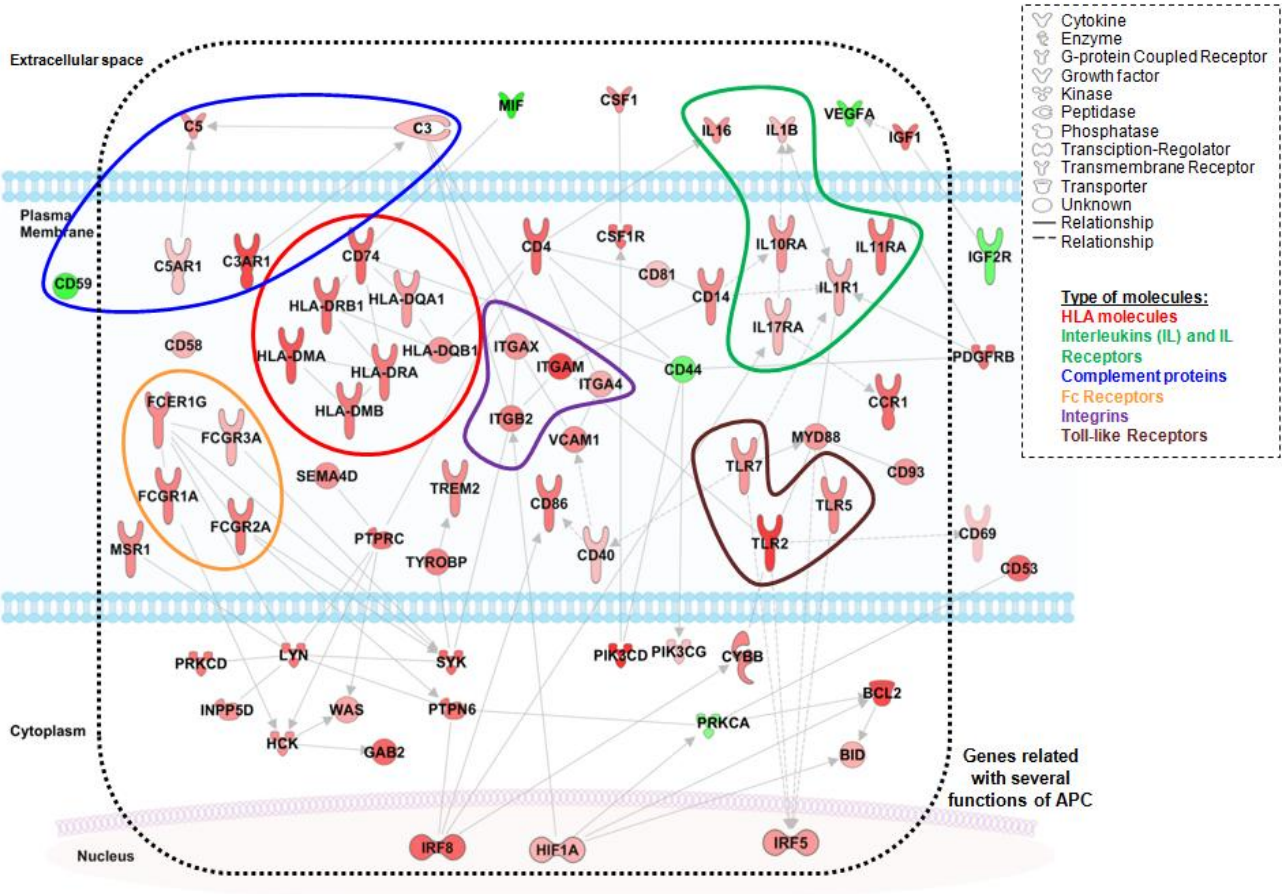

Figure 21. Schematic representation of the functional impact of GEP of meningiomas with isolated monosomy 22/del(22q). The scheme was built based on the results obtained through the analysis of GEP performed with the Ingenuity Pathway Analysis software and it shows increased expression of several inflammatory genes, particularly genes involved in antigen presenting cell functions, among cases with isolated monosomy 22/del(22q). Such genes include HLA and HLA-associated molecules (*HLA-DMA*, *HLA-DMB*, *HLA-DRA*, *HLA-DRB1*, *HLA-DQA1*, *HLA-DQB* and *CD74*), cytokines (*IL16*, *IL1B*, *IL1R1*, *IL10RA*, *IL11RA* and *IL17RA*), growth factors and growth factor receptors (*CSF1*, *CSF1R*, *IGF1*, *IGF2R*, *VEGF* and *PDGFRB*), complement proteins (*C5*, *C3*, *C3AR1*, *C5AR1* and *CD59*), immunoglobulin Fc (FcIg) receptors (*FCGR1A*, *FCGR2A*, *FCGR3B* and *FCER1G*) and the *CCR1* chemokine receptor, integrins (*ITGAM*, *ITGAX*, *ITGA4* and *ITGB2*) and other adhesion molecules (*VCAM1*, *CD44*, *CD53*, *CD58*, *CD81* and *CD93*), immune co-stimulatory molecules (*CD4*, *CD40* and *CD86*), toll-like receptors (*TLR2*, *TLR5* and *TLR7*) and TLR-associated molecules (*CD14* and *MYD88*), in addition to phosphoinositide-3-kinases (*PIK3CG* and *PIK3CD*) and other kinases (*PRKCA*, *PRKCD*, *SYK*, *LYN* and *HCK*), tyrosine phosphatases (*PTPRC* and *PTPN6*), and apoptotic proteins (*BCL2* and *BID*), and other signaling molecules (*CD69*, *CYBB*, *GAB2*, *HIF1A*, *INPP5D*, *IRF5*, *IRF8*, *MIF*, *MSR1*, *SEMA4D*, *TREM2*, *TYROBP* and *WAS*).

Noteworthy, a significant correlation was observed in those 13 meningioma samples in which GEP and flow cytometry immunophenotyping were performed in parallel, for the percentage of inflammatory and other immune cells infiltrating the tumor and the mRNA levels of proteins specifically expressed by these cells (e.g. TiMa) such as HLA-DR (r^2=0.8; p<0.001), CD14 (r^2=0.8; p<0.001), Cybcl2 (r^2=0.7; p=0.01), CD53 (r^2=0.7; p=0.01), CD37 (r^2=0.7; p=0.01), CD99 (r^2=0.6; p=0.02), CD45 (r^2=0.6; p=0.03), CD16 (r^2=0.6; p=0.04), CyD68 (r^2=0.6; p=0.04), and HLA-I (r^2=0.6; p=0.04).

4. Discussion

According to the World Health Organization, meningiomas are mostly classified as grade I benign tumors [3]; however, grade I meningiomas are genetically very heterogeneous [73]. Accordingly, distinct cytogenetic profiles have been identified in meningiomas, which include (i) diploid tumors, (ii) tumors showing isolated monosomy 22/del(22q), (iii) del(1p36) alone, (iii) isolated loss of a sex chromosome, and (iv) meningiomas with complex karyotypes in the absence or (v) presence of del(1p36) and/or monosomy 14. From the prognostic point of view, meningiomas which have complex karyotypes, particularly those carrying del(1p36) and/or monosomy 14, display a significantly worse outcome, whereas diploid tumors and cases with isolated monosomy 22/del(22q) have a particularly good prognosis [73], as confirmed also in our series.

At present, the specific factors that contribute to the better outcome of -22/22q$^-$ cases remain to be elucidated. Previous studies have claimed that monosomy 22/del(22q) is frequently associated with NF2 mutation, the later potentially representing the first chromosomal alteration to occur in meningiomas; if this hypothesis holds true, cases carrying an isolated loss of chromosome 22 could represent the earliest stage of neoplastic transformation in meningiomas [3]. However, more recent studies in which the intratumoral patterns of cytogenetic evolution have been analyzed in detail indicate that this is probably not the case; more likely, loss of chromosome 22/*NF2* mutation represents one of multiple pathways of intratumoral clonal evolution occurring in benign grade I meningiomas [224]. In line with this hypothesis, Clark et al. [106] have recently reported distinct genome profiles of meningiomas based on the presence *vs.* absence of *NF2* mutations, non-*NF2* mutated meningiomas frequently showing mutations in other genes (e.g. *TRAF7*, *KLF4*, *AKT1* and *SMO*).

In turn, it should be taken into account that tumor behavior depends not only on tumor cytogenetics, but also on the tumor microenvironment, including surrounding cells which may either support tumor growth or control the disease [448]. In this regard, the potential role of immune cells infiltrating the tumor has become particularly relevant, as the presence of inflammatory and both cytotoxic and regulatory cells has been correlated with the behavior of the disease (e.g. patient outcome) in multiple different tumor types [446]. In the present study, we show a clear association between the levels of immune/inflammatory cells infiltrating meningiomas and tumor cytogenetics. Tumor infiltration by immune/inflammatory cells had already been shown to be associated in meningiomas with both tumor grade and the histopathological subtypes [272, 276]. By contrast, to the best of our knowledge, this is the first report which shows a clear relationship between inflammatory/immune infiltrates and

tumor cytogenetics in meningiomas. Overall, meningiomas carrying isolated monosomy 22/del(22q) showed significantly greater numbers of TiMa infiltrating the tumor, together with a more pronounced activation profile of immune cells, as reflected by greater percentages of $CD69^+$ and $CD63^+$ TiMa and/or lymphocytes [449, 450], *vs.* cases with either a diploid or a complex iFISH karyotype. In addition, HLA-$DR^+CD14^+CD45^+CD68^+$ TiMa from patients with isolated monosomy 22/del(22q) also showed higher levels of expression of CD16, an FcγRIII receptor typically absent in recently produced blood monocytes, but expressed during macrophage maturation in peripheral tissues (e.g. alveolar and pleural macrophages) [451, 452]. From a functional point of view, expression of CD16 enables macrophages to carry out antibody-dependent cell-mediated cytotoxic functions, which would make them also more efficient phagocytic cells [453]. Altogether, these results suggest that in addition to the greater numbers of TiMa infiltrating the tumor, TiMa from meningiomas carrying monosomy 22/del(22q) alone would also show a more activated and functionally matured phenotype. In this regard, TiMa of both -22/$22q^-$ and cytogenetically complex meningiomas also showed higher expression of both the CD44 and CD9 adhesion molecules *vs*. diploid cases. CD44 is a cell-adhesion molecule which is expressed by macrophages [454], and has been previously reported to be up-regulated in tumor-associated macrophages, playing a role in their recruitment and activation [397, 455]. Similarly, the CD9 tetraspanin has also been reported to promote activation of macrophages through its functional association with Fcγ receptors [456]. These observations further support a central role for TiMa in controlling tumor growth, as well as in promoting homing/chemoattraction of inflammatory and other immune cells to the tumor, among meningiomas with isolated monosomy 22/del(22q), which could potentially contribute to explain the better outcome of this specific subgroup of meningioma patients *vs.* cases with complex karyotypes.

In line with this hypothesis, meningiomas with isolated monosomy 22/del(22q) also displayed a greater infiltration by NK cells and lymphocytes expressing the CD69 early-activation antigen. Although NK cells and $CD69^+$ activated lymphocytes only represented a small fraction of all infiltrating cells they may also contribute to immune surveillance and to the elimination of tumor cells and thus, to control tumor growth through direct cytotoxic mechanisms cooperating with those of tissue macrophages [335].

To further investigate the molecular mechanisms involved in inflammatory and immune responses in those tumors carrying isolated monosomy 22/del(22q) *vs.* other meningiomas, we further analyzed the GEP of tumor samples from the distinct cytogenetic subgroups of meningiomas. As expected, meningiomas with isolated monosomy 22/del(22q) typically showed a GEP associated with an increased inflammatory and immune response

consisting of greater expression of genes involved in antigen presentation (e.g. HLA and HLA-associated molecules), phagocytosis (Fc receptors) and cell activation/cell signaling (e.g. immune co-stimulatory molecules, toll-like receptors and inflammatory cytokines), when compared to tumors with diploid and complex karyotypes.

So far, two distinct populations of functionally polarized macrophages have been described, which are generated depending on the cytokines present in the tissue microenvironment: classical M1 macrophage which develop under the influence of LPS and IFN-γ, produce pro-inflammatory cytokines (e.g. IL-12, IL-1, and IL-6), mediate resistance to pathogens and contribute to tissue destruction, and; M2 macrophages, developed under the influence of IL-4 and IL-10, which produce anti-inflammatory cytokines (e.g. IL-10 and TGF-β), promote tissue repair and remodeling and support tumor progression [286]. Despite lacking specific markers, M1 macrophages express receptors like CD16, CD32, CD64 and CD86, while M2 macrophages are characterized by abundant levels of CD163 and CD206. Although until now there is no information about the type of macrophages that infiltrate meningiomas, the higher expression levels of CD16 (FCGR3A) found here both at the protein and mRNA levels, together with the increased mRNA levels of *CD86*, *CD32* (*FCGR2A*) and *CD64* (*FCGR1A*) observed in meningiomas with monosomy 22/del(22q) alone, support an M1 vs M2 polarization of macrophages in this subgroup of meningiomas and consequently also, a more favorable anti-tumoral microenvironment. In addition, the higher expression levels of HLA-DR, a marker commonly used in TiMa from solid tumors to indicate an M1-phenotype [404, 405, 457, 458], also support this notion. In line with this, NK cells have been reported to play an indirect role in redirecting macrophage activation toward the M1 phenotype [286, 459], NK cells being also found at higher numbers in our series of meningiomas with an isolated -22/22q$^-$ karyotype. Similarly, higher levels of expression of *IRF5* and *IRF8*, but not *IRF4*, were reported as part of the GEP characteristic of -22/22q$^-$ meningiomas; while *IRF5* production has been shown to play a critical role in M1 macrophage polarization [460], IRF4 stimulates expression of M2 macrophage markers [461]. Altogether these results support a predominant M1 polarization of macrophages in meningiomas with isolated monosomy 22/del(22q) and potentially also their better prognosis *vs.* other cytogenetic subtypes of meningiomas (e.g. cases with complex karyotypes). However, further investigations on this matter are needed to evaluate in more detail the functionality and phenotype of TiMa in meningiomas as regards M1 *vs.* M2 polarization.

In this regard, we further analyzed in a series of 33 cases the expression of the CD206 M2 phenotype-associated marker. Our preliminary results show that despite no significant differences were found between the cytogenetic subgroups for the CD206 levels on tumor-

infiltrating TiMa, higher CD206 expression was found in meningiomas with complex karyotypes associated with high percentages of TiMa infiltration *vs.* cases with isolated monosomy 22/del(22q). These results suggest a functional polarization of TiMa towards an M2-phenotype in cases with complex karyotypes and high TiMa infiltration levels, in contrast to meningiomas with isolated chromosomal 22 losses whose TiMa displayed an M1 functional phenotype. As far as we know, this is the first data on the M1/M2 phenotype of TiMa in meningiomas. Of note, such M2 TiMa phenotype among the subset of meningiomas with complex karyotypes and high levels of TiMa infiltration was also associated with increased numbers of Tregs and low numbers of NK-cells which have both been also associated with an M2-phenotype and suppression of immune responses at the tumor microenvironment [286]. In addition, infiltration by M2-polarized macrophages and Tregs has also been reported to be involved in tumor recurrence and poorer prognosis, as well as failure of vaccines administrated after surgery [462]. Altogether these results suggest the potential relevance of both the type of immune cells, and the level of infiltration of such cells, in the regulation of meningioma growth and tumor immunogenicity. Further investigations about the functional behavior of infiltrating macrophages in meningiomas are needed to confirm these hypotheses.

Whether or not the inflammatory responses in meningiomas are directly determined by the loss of expression in tumor cells of genes specifically coded in chromosome 22/22q, also deserves further investigation. Despite this, it should be noted that the most significant immune response-associated gene coded in chromosome 22, which was lost in this cytogenetic subgroup of meningiomas, is the *MIF* gene. MIF was originally identified as a T-cell-derived factor responsible for the inhibition of macrophage migration [463]. However, nowadays MIF has been recognized to act as a pro-inflammatory cytokine which is both involved in inflammatory and immune responses, as well as in tumor cell growth and invasiveness [463, 464]. In this regard, recent studies indicate that MIF protein levels are elevated in cancer patients [464, 465] and that MIF expression directly correlates with stage, metastatic spread, disease-free survival and tumor-associated neovascularization in e.g. lung, prostate, breast and gastric cancer, as well as glioma patients [464, 466-470]. Thus, loss of MIF in meningiomas with isolated monosomy 22/del(22q) may also play an important role in determining the more indolent behavior and the good prognosis of this subgroup of meningioma patients.

In summary, our results indicate that an increased infiltration of the tumor by tissue macrophages, NK cells and activated lymphocytes in meningiomas, is specifically associated with cases carrying an isolate monosomy 22/del(22q) and a subset of meningiomas displaying complex karyotypes. In contrast with the former tumors, TiMa infiltrates in the latter cases

seem to have undergone a functional shift towards an M2 *vs.* M1 phenotype, in association with immune suppression by higher levels of infiltrating Tregs and lower NK-cell numbers, potentially associated with an increased pro-tumoral inflammatory response. The precise clinical impact of these findings deserves further investigations.

Chapter VI

The protein expression profile of meningioma cells is associated with distinct cytogenetic tumor subgroups

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1. Abstract

Limited information exists about the impact of cytogenetic alterations on the protein expression profiles of individual meningioma cells and its association with the clinico-histopathological characteristics of the disease. Here, we investigate the potential association between the immunophenotypic profile of single meningioma cells and the most relevant features of the tumor. Multiparameter flow cytometry (MFC) was used to evaluate the immunophenotypic profile of tumor cells (n=51 patients) and the Affymetrix U133A chip was applied for the analysis of the gene expression profile (n=40) of meningioma samples, cytogenetically characterized by interphase fluorescence in situ hybridization. Overall, a close association between the pattern of protein expression and the cytogenetic profile of tumor cells was found. Thus, diploid tumors displayed higher levels of expression of the CD55 complement regulatory protein, tumors carrying isolated monosomy 22/del(22q) showed greater levels of bcl2 and PDGFRβ and meningiomas carrying complex karyotypes displayed a greater proliferation index and decreased expression of the CD13 ectoenzyme, the CD9 and CD81 tetraspanins, and the Her2/neu growth factor receptor. From the clinical point of view, higher expression of CD53 and CD44 was associated with a poorer outcome. In summary, here we show that the protein expression profile of individual meningioma cells is closely associated with tumor cytogenetics, which may reflect the involvement of different signaling pathways in the distinct cytogenetic subgroups of meningiomas, with specific immunophenotypic profiles also translating into a different tumor clinical behavior.

2. Introduction

Meningiomas consist of a heterogeneous group of central nervous system (CNS) tumors both on histopathological and genetic/molecular grounds [3, 6, 471]. Most meningiomas show a benign clinical behavior and patients are cured after complete surgical resection of the tumor. However, up to 20% of the cases will show tumor recurrence, which leads to an increased morbidity and mortality [6, 60]. For decades now, it is well established that grade II (e.g. atypical) and grade III (e.g. anaplastic) meningiomas show higher recurrence rates and a poorer prognosis, compared to grade I tumors [3, 6]. Despite this, in absolute numbers, the majority of recurrences observed among meningioma patients still occur in histologically benign/grade I tumors [60].

In recent years, evidences have accumulated which show an association among histologically benign/grade I meningiomas, between complex tumor karyotypes (≥2 genetic alterations), particularly those that include monosomy 14, and a shorter patient relapse-free survival (RFS) [63, 73, 79, 471]. Although distinct cytogenetic subtypes of meningiomas are associated with specific histopathological subtypes and unique gene expression profiles (GEP), to the best of our knowledge, no study has been reported so far in which the pattern of expression of a broad panel of proteins has been analyzed in meningiomas to determine whether the immunophenotypic profile of single cells from individual tumors is associated with the most relevant features of the disease, including tumor histopathology and cytogenetics, as well as patient outcome. In this regard, we have recently shown that multiparameter flow cytometry (MFC) immunophenotyping is a well-suited technique for the evaluation of the pattern of (quantitative) expression of relatively large numbers of tumor-associated proteins in individual tumor cells, when an appropriate marker combination is used for exclusion of other types of non-neoplastic cells (e.g. inflammatory cells) infiltrating the tumor [445].

In this study, we analyzed the pattern of expression of a large panel of markers by MFC, in 51 meningiomas. Our ultimate goal was to determine the potential association between the immunophenotypic profile of individual tumor cells and the clinical, histopathological and cytogenetic features of the disease, as well as patient outcome. Overall, our results show that a close association exists in meningiomas between the pattern of protein expression and the cytogenetic profile of tumor cells, pointing out the involvement of different pathogenetic mechanisms associated with unique protein expression profiles, in different cytogenetic subgroups of meningiomas.

3. Results

3.1. Immunophenotypic profile of meningioma cells and its association with the clinico-biological and cytogenetic features of the disease.

Overall, tumor cells systematically displayed high reactivity for the CD9, CD63 and CD81 tetraspanin molecules, the CD55/CD59 complement regulatory proteins, HLA-I and the CD13 ectoenzyme (Supplementary Table 2). The other markers investigated showed more variable patterns of expression. Some were detected in most cells from the majority of cases – PDGFRβ (77±28% of PDGFRβ$^+$ cells from 96% of cases), IGFR (73±25% of IGFR$^+$ cells from 96% of cases), HER2/neu (73±26% positive cells from 96% of cases), EGFR (69±24% of EGFR$^+$ cells from 78% of cases), CD38 (66±27% of CD38$^+$ cells from 86% cases), bcl-2 (65±24% positive cells from 86% of cases), CD14 (76±19% positive cells from 76% of cases) and HLA-DR (69±23% positive cells from 80% of cases) -, while other proteins were present in a lower percentage of cases (62±24%, 61±22%, 60±21% and 51±21% of CD53, CD58, CD99 and CD2 positive cells in 63%, 59%, 47% and 22% of cases, respectively). Additionally, meningioma cells showed a mean (± 1 SD) proliferation index by multiparameter flow cytometry of 10%±6%, with a greater variability among different cases (wider range of percentages) than when assessed in parallel, as percentage of MIB-1$^+$ cells by immunohistochemistry, in a subset of 10 cases (data not shown).

From the clinical point of view, female meningiomas and convexity/parasagittal tumors showed greater expression of Cybcl2 (p=0.03 and p=0.01, respectively). Parasagittal tumors also showed higher expression of CD63 *vs.* all other meningiomas (p≤0.04), whereas spinal tumors presented lower reactivity (p≤0.03 *vs.* intracranial tumors) for the IGFR growth factor receptor. Additionally, fibroblastic meningiomas showed higher bcl2 levels/tumor cell *vs.* other histological subtypes (p=0.002), while CD99 expression was greater in transitional *vs.* meningothelial meningiomas (p=0.004) and the reactivity for PDGFRβ was significantly lower among high grade meningiomas (p=0.04). Presence of moderate to severe edema was associated with higher CD38 expression (p=0.02) and lower reactivity for HLA-DR (p=0.02).

Despite all the above associations, protein expression profiles of tumor cells from meningiomas were most strongly associated with tumor cytogenetics (Table 11). Thus, meningiomas with complex karyotypes showed decreased expression of the CD55 complement regulatory protein (p=0.01 *vs.* diploid tumors), the CD9 (p<0.001 *vs.* all other groups) and CD81 (p<0.03 *vs.* all other groups) tetraspanins, the CD13 ectoenzyme (p<0.04 *vs.* all other groups) and the HER2/neu growth factor receptor (p<0.02 *vs.* all other groups) (Table 11 and Figure 22A). In turn, cases with isolated monosomy 22/del(22q) displayed a higher

reactivity for the PDGFRβ receptor (p<0.01 *vs.* diploid and complex tumors; Table 11 and Figure 22B) and bcl2 (p<0.005 *vs.* diploid and complex tumors; Table 11 and Figure 22C). In addition, a progressively higher PI was found from diploid tumors, to cases with isolated -22/22q$^-$ and meningiomas with complex karyotypes (p<0.003).

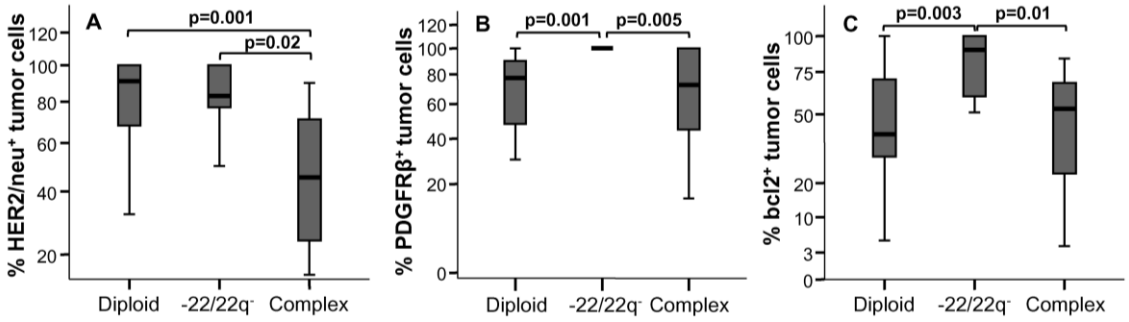

Figure 22. Distribution of Her2/neu$^+$, PDGFRβ$^+$ and bcl2$^+$ tumour cells in meningiomas grouped according to their cytogenetic profile. Expression of surface membrane Her2/neu, PDGFRβ and cytoplasmic bcl2, as percentage of all CD44$^+$CD45$^-$ tumour cells, is shown in panels A, B and C, respectively; for all other markers investigated, no significantly different percentages of positive cells were found among the distinct cytogenetic groups of meningiomas. Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively.

Unsupervised hierarchical clustering analysis based on the immunophenotypic features of tumor cells, revealed two clearly distinct subgroups of meningiomas (Figure 23). Interestingly, while one group included mostly (21/25 cases, 84%) patients with diploid meningiomas (11/17 diploid cases; 65%) or tumors carrying an isolated cytogenetic alteration (10/17 tumors with monosomy22/del(22q); 60%), the other group comprised almost all patients with a complex karyotype (12/16 cases, 75%). Of note, the majority of grade II/III meningioma samples (4/5 cases) were included in this latter group. The former group was characterized by a low PI together with a higher reactivity for CD9, CD55, CD81, CD13, PDGFRβ and HER2/neu, while the latter group displayed higher PI and lower levels of expression of the above referred markers (Figure 23).

Concerning patient outcome, although recurrent tumors showed higher levels of expression of HLA-DR (p=0.02), CD44 (p=0.01) and CD53 (p=0.006), only the two latter markers retained a significant adverse impact on RFS (p=0.01 and p=0.04, respectively; Figure 24), in addition to tumor grade (p<0.001) and cytogenetics (p=0.003; Supplementary Figure 1). Multivariate analysis for RFS, including those variables which showed prognostic impact in the univariate analysis, showed that tumor grade was the only variable retaining an independent prognostic value (p=0.03) in this group of meningiomas. Of note, co-expression of both the

CD44 and CD53 markers at higher levels/tumor cell was associated with a particular adverse impact on patient RFS (p=0.001; Figure 24C).

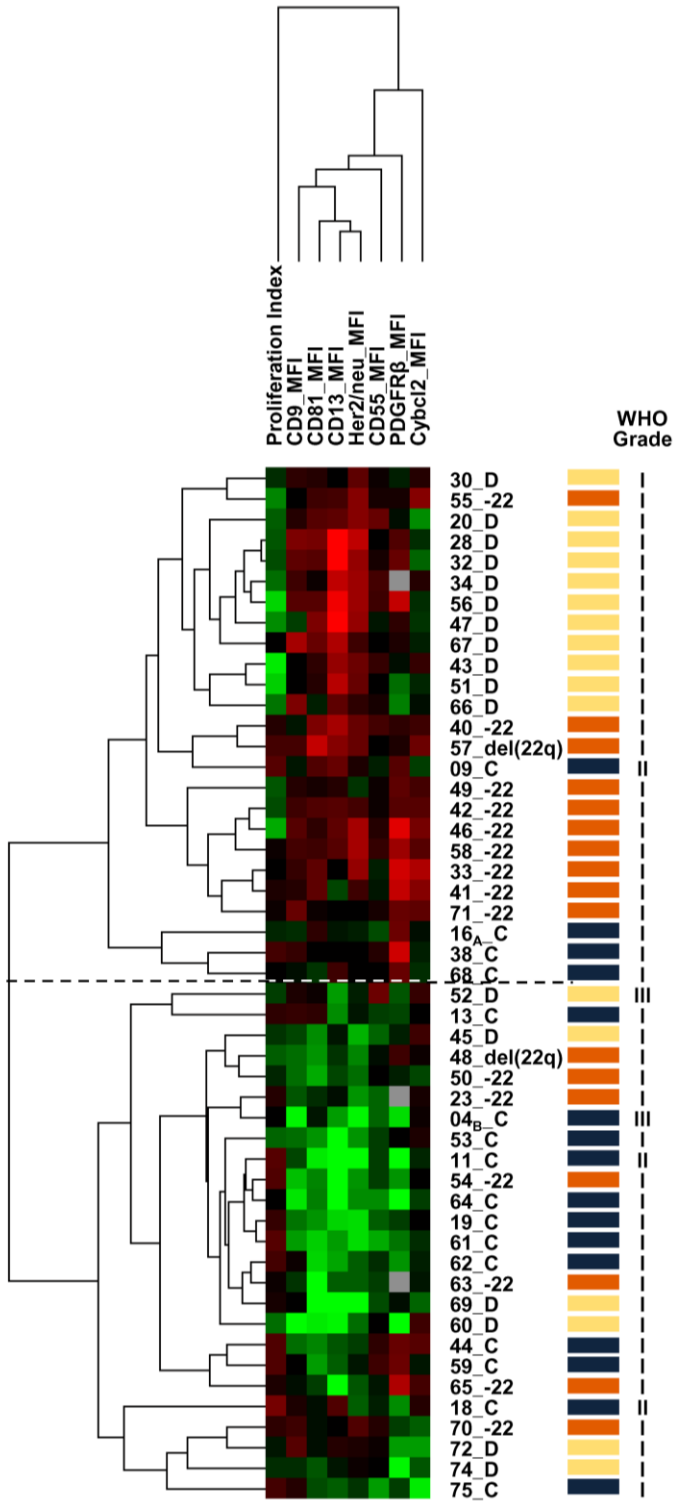

Figure 23. Hierarchical clustering analysis of meningioma samples based on the immunophenotypic characteristics and cell cycle distribution of tumour cells and its relationship with the different cytogenetic and WHO grade subgroups of the disease. Results are shown in a matrix format where each column represents a single variable and each row represents a different meningioma sample (rows identified with a 'D', '22' and 'C' correspond to diploid, isolated monosomy 22/del(22q) and complex iFISH karyotype meningiomas, respectively). Normalized values are represented by a colour scale where red and green colours reflect values above and below the median values obtained for each variable, respectively. On the right side of the figure, the hierarchical clustering of samples obtained is shown where most of the tumours tend to be grouped by protein expression profiles according to their iFISH cytogenetic pattern.

Table 11. Association between the clinico-biological and cytogenetic characteristics of meningioma patients and both the immunophenotype and proliferation index of tumour cells.

	PI	Immunophenotypic Markers													
		HLA-DR	CD55	CD9	CD53	CD63	CD81	CD44	CD99	CD13	CD38	Cybcl2	HER2/neu	IGFR	PDGFRβ
Age															
<50 (n=13)	8±6	3181±2626	4580±2141	29268±23183	485±406	3964±1800	20900±12571	5332±6785	362±288	24018±26393	2169±2312	1128±760	2375±1912	1283±693	2663±2833
>50 (n=38)	11±6	2404±2974	5189±2392	24705±15454	564±950	3334±1587	15903±14881	6631±9990	256±219	16771±23806	1707±1905	831±496	1743±1599	1244±1011	2167±2081
Sex												†			
Female (n=37)	9±6	2479±1999	4967±1911	25168±10492	488±497	3454±1675	16580±11889	5764±7887	267±199	18954±24673	1757±1726	987±606	1995±1796	1253±936	2544±2548
Male (n=14)	12±6	2928±4556	5208±3260	27673±20586	692±1418	3602±1633	18754±19991	7718±12351	325±331	17733±24660	2003±2670	695±466	1664±1390	1258±964	1640±1143
Tumour localization						† #						† &		† ‡	
Convexity/Parasagittal (n=31)	12±6	2999±3414	4819±2358	24220±17369	628±1009	3994±1848	18348±15947	6016±8072	312±272	14697±18985	1917±2314	1047±611	1663±1520	1194±789	2650±2457
Cranial base/Tentorial/ Intraosseous (n=17)	8±5	1934±1501	5532±2418	30239±18705	472±496	2852±853	16696±11823	7556±11775	239±178	27638±32384	1790±1511	675±481	2559±1928	1542±1127	1547±1819
Spinal (n=3)	7±3	2284±2940	4427±1251	18121±11182	78±19	1980±378	7795±8811	2122±1817	230±211	8036±4918	1062±803	770±459	683±171	254±160	1989±1298
Edema		†									†				
No/Light (n=30)	10±5	3321±3398	4920±2242	27952±18972	621±1017	3339±1624	18920±16117	5210±7352	323±281	16688±19546	1239±1180	1006±652	2048±1689	1255±948	2269±2233
Moderate/Severe (n=21)	11±7	1575±1470	5196±2485	22890±15383	434±502	3718±1699	14687±11352	7857±11417	225±154	21377±30402	2661±2599	766±438	1699±1705	1254±936	2325±2377
Tumour grade															†
Grade I (n=46)	9±6	2545±2922	5043±2154	26711±18074	536±872	3332±1488	17420±14862	5836±9203	295±246	19610±25354	1761±1914	920±605	2032±1719	1269±975	2465±2324
Grade II/III (n=5)	15±8	3123±2740	4951±3915	18109±10645	612±545	4995±2445	14941±9548	10571±9333	175±153	9499±10574	2410±2904	788±298	727±661	1116±423	854±1081
Tumour histopathology									† ‡			† #			†&
Meningothelial (n=14)	11±7	1955±1536	4878±2355	26617±17020	437±485	3328±2003	11197±7620	6565±9419	154±91	11801±13390	2172±2948	759±383	1548±1381	1359±1006	1663±2005
Transitional (n=16)	10±5	2960±4201	5084±2402	30896±21952	778±1361	3608±1052	22973±19520	8363±12310	402±312	29518±35208	1582±1310	783±513	2427±1963	1528±1187	2641±2171
Psammomatous (n=7)	8±4	2464±2373	5163±2196	23469±14163	274±256	2849±1021	13987±11078	2230±1875	314±247	18830±23615	850±519	796±443	1953±2039	794±590	3276±2843
Fibroblastic (n=7)	10±5	3278±2553	5117±1860	24484±15990	540±444	3395±1852	22399±14160	3341±3834	358±199	12446±12402	2114±1237	1688±848	2246±1634	1095±649	3466±2783
Other (n=7) **	12±9	2539±2455	5017±3199	16658±10770	496±492	4315±2313	13855±9527	8085±8725	162±134	13303±16463	2369±2551	813±309	1031±1041	1041±475	707±929
iFISH karyotype *	††† §		† #	† &			† &			†† &		†† ‡	†† &		† ‡
Diploid (n=17)	5±3	1788±1178	6174±2646	34809±21557	434±497	2906±859	20467±12989	8112±11528	283 ± 251	36089±34715	1909±1727	671±357	2779±1838	2779±1838	1571±1699
Monosomy 22/del(22q) (n=17)	9±5	3977±4174	5092±1700	26848±14223	724±1280	3353±1549	21395±17872	3775±6389	394 ± 280	13009±10095	1353±1055	1366±719	2185±1723	2185±1723	3552±2659
Complex (n=16)	15±6	2126±2066	3913±2060	16609±10480	496±535	4375±2072	10098±8276	7417±9182	179 ± 114	7162±7140	2313±2895	696±277	766±492	766±492	1836±1998
Relapse		†			††			††							
No (n=47)	10±6	2145±1814	5179±2350	26420±17807	411±445	3359±1514	15713±12166	5587±9027	263±219	18852±25259	1896±2065	906±587	1938±1726	1254±964	2320±2349
Yes (n=4)	13±6	7968±6816	3329±1099	19379±15487	2103±2293	5093±2548	34372±27326	14684±8316	512±384	15875±12307	980±662	918±587	1503±1235	1256±523	2010±1361

Results are expressed as mean MFI ± one standard deviation (SD); markers that showed no statistical significance (HLA-I, CD59, CD2, CD58, EGFR and CD14) are not shown.
PI: Proliferation Index (%S+G2/M cells).
P-value (Kruskal-Wallis test): † 0.01<p<0.05; †† 0.001<p<0.01; ††† p<0.001.
Significant differences (Mann-Whitney U test) for:
- tumour localization: #, convexity/parasagittal *vs.* others; ‡, spinal *vs.* others; &, convexity/parasagittal *vs.* cranial base/tentorial/intraosseous.
- tumour histopathology: #, fibroblastic *vs.* all others; ‡, transitional *vs.* meningothelial; &, meningothelial/ transitional/ fibroblastic *vs.* other.
- iFSH karyotype: #, D *vs.* C; ‡, -22 *vs.* others; &, C *vs.* others; §, all groups.

*The only patient with isolated del(1p36) was excluded from the analysis; ** Includes 3 atypical, one secretory, one rhabdoid, one angiomatous and one papillary tumour.

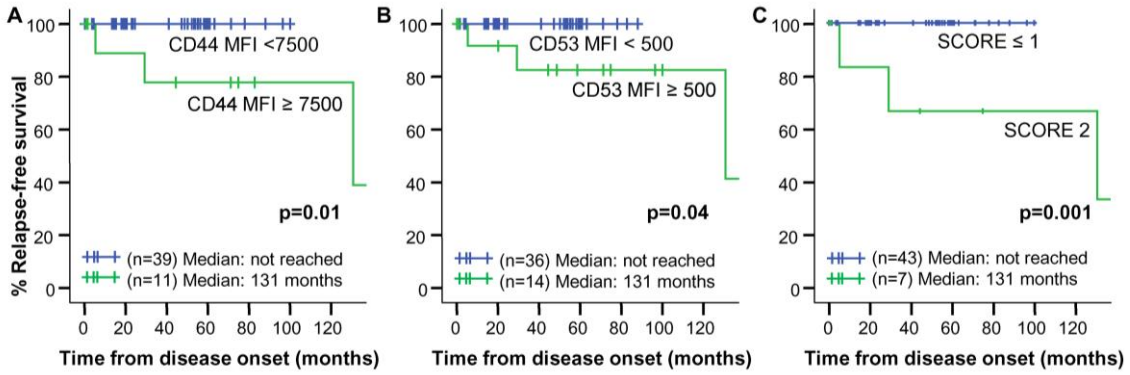

Figure 24. Impact of the pattern of protein expression of tumour cells on relapse-free survival of meningioma patients. Relapse-free survival curves of meningioma patients classified according to the levels of expression of CD44 (n=50; panel A) and CD53 (n=50; panel B) per tumour cell, are shown. In panel C the impact on relapse-free survival of a score built on the basis of the expression of both CD44 and CD53 is shown: score 0 and 1 were assigned for tumour samples expressing none or only one of the markers and score 2 was assigned to cases showing co-expression of both CD53≥500 (MFI) and CD44≥7500 (MFI). All other markers analyzed did not show an impact on patient relapse-free survival. MFI: mean fluorescence intensity per tumour cell (arbitrary fluorescence units scaled from 0 to 262144).

3.2. Relationship between mRNA and protein expression levels among the different cytogenetic subgroups of meningiomas.

The relationship between mRNA and protein expression levels was investigated in a subset of 13 meningioma samples in which both sets of parameters were simultaneously analyzed. Overall, a similar pattern of expression of CD13 and Cybcl2 was observed at the mRNA and protein levels among distinct cytogenetic subgroups of meningiomas (Figure 25A and 25B), with a high correlation coefficient between mRNA and protein expression (r^2=0.9, p=0.001 and r^2=0.5, p=0.1, respectively). In line with flow cytometry results, *CD13* mRNA was also decreased in tumors with a complex karyotype *vs.* diploid meningiomas (p=0.01; Figure 25A). In turn, cases with isolated monosomy 22/del(22q) showed higher *BCL2* mRNA expression *vs.* all other tumors (p≤0.03; Figure 25B). Of note, despite a lower correlation coefficient, the PDGFRβ receptor also showed higher expression at both the mRNA and the protein levels among tumors carrying isolated monosomy 22/del(22q) (p≤0.03; Figure 25C). Conversely, an inverse correlation between both the HER2/neu and CD55 mRNA *vs.* protein levels was observed (r^2=-0.7, p=0.006 and r^2=-0.5, p=0.008, respectively), with significantly different amounts of protein/cell, but similar mRNA expression levels in the distinct cytogenetic subgroups of meningiomas (Figure 25D and 25E, respectively). Finally, no significant correlation was found between the mRNA expression profiles and the protein levels of the CD9 and CD81 tetraspanin molecules (Figure 25F and 25G, respectively).

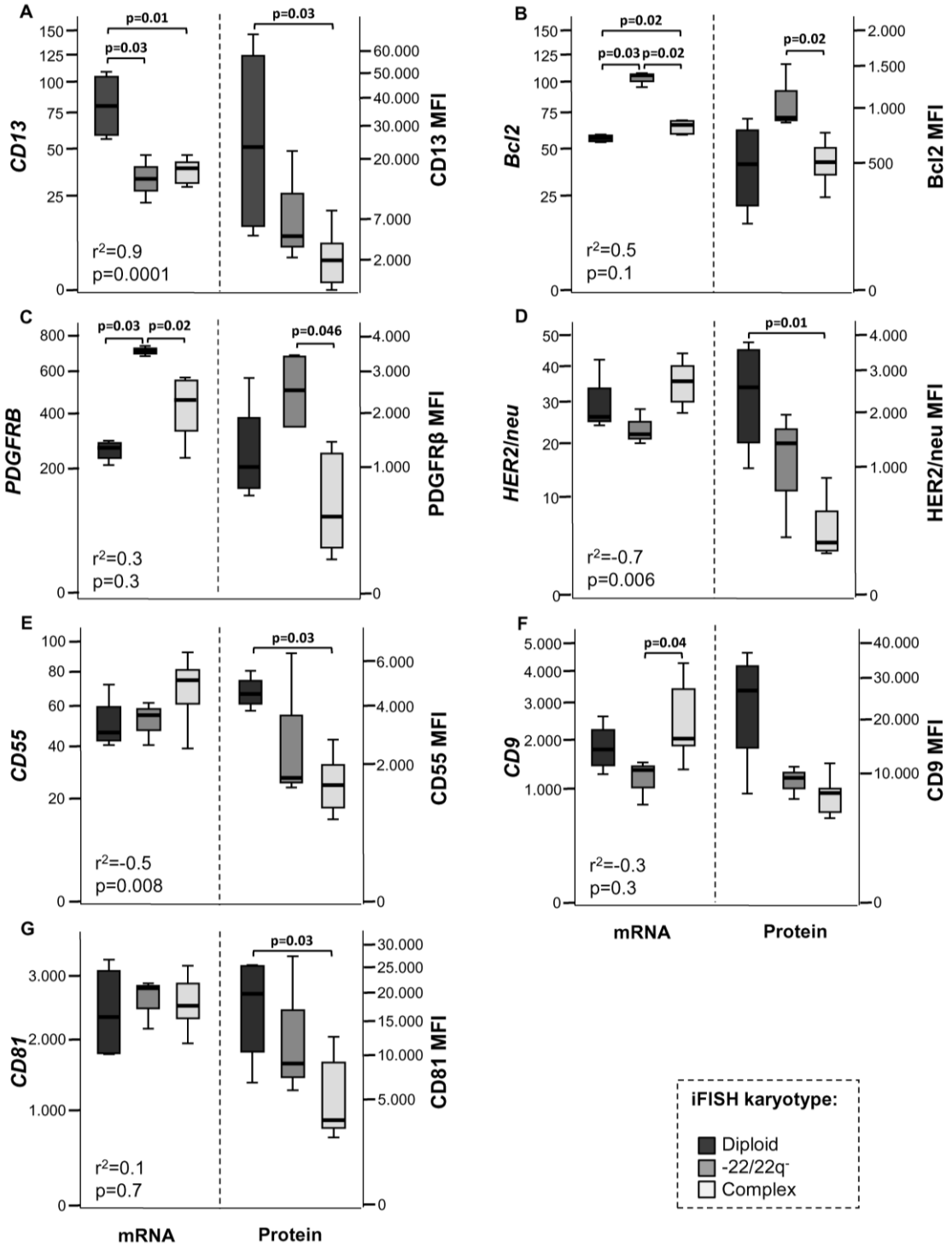

Figure 25. Relationship between the mRNA and protein expression levels of individual tumour cells in meningiomas grouped according to their iFISH cytogenetic profile. The amount of expression of CD13 (panel A), Cybcl2 (panel B), PDGFRβ (panel C), HER2/neu (panel D), CD55 (panel E), CD9 (panel F) and CD81 (panel G), are shown both at the mRNA (arbitrary fluorescence units) and at the protein level (MFI, mean fluorescence intensity) for cases in which both measures were performed simultaneously (n=13). Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles, respectively. mRNA probe set numbers represented were selected from the U133A Affymetrix microarrays as follows: *CD13* 202888_s_at; *BCL2* mean of 203684_s_at / 203685_at / 207004_at / 207005_s_at; *PDGFRB* 202273_at; *HER2/neu* 210930_s_at; *CD55* mean of 201925_s_at / 201926_s_at; *CD9* 201005_at; *CD81* 200675_at.

Based on the differential GEP found for the three cytogenetic subgroups of meningiomas for the immunophenotypic markers here analyzed, we built a schematic map representation of those intracellular pathways in which the cell surface and cytoplasmic molecules that showed unique patterns of expression in individual cytogenetic subgroups, are involved (Figure 26). High *CD13* (*ANPEP*) mRNA and protein expression was characteristic of diploid meningioma samples, while higher *PDGFRB* and *BCL2* mRNA and protein levels were usually observed in meningiomas carrying monosomy 22/del(22q); in turn, tumors with complex karyotypes typically showed higher levels of *CD44* mRNA (but not protein) expression, supporting the involvement of different signaling pathways in the distinct cytogenetic subgroups of meningiomas.

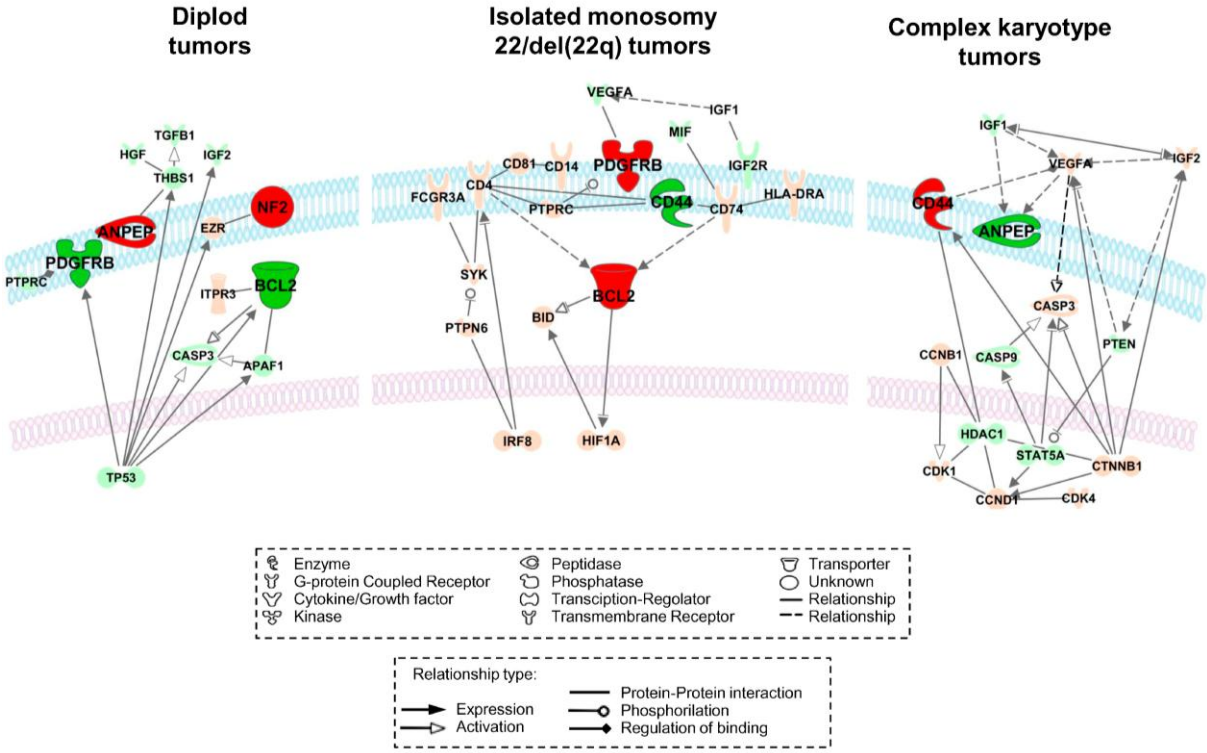

Figure 26. Schematic representation (Ingenuity Pathway Analysis software) of those signalling pathways for which distinct gene/protein expression profiles (GEP) were observed in meningioma tumour cells carrying different iFISH cytogenetic profiles (n=40 tumours)**.** Genes highlighted in red correspond to overexpressed genes and those highlighted in green are underexpressed genes in the corresponding iFISH cytogenetic subgroup of meningiomas *vs.* other cytogenetic subgroups.

4. Discussion

In this study we analyzed the pattern of expression of a relatively large panel of proteins in single tumor cell suspensions from meningioma samples, using MFC. To the best of our knowledge, this is the first study to provide detailed immunophenotypic profiles of individual meningioma cells. Our results showed a clear association between the pattern of expression of several markers and tumor cytogenetics. As cytogenetically heterogeneous tumors [3, 471], meningioma samples showed three major cytogenetic profiles which corresponded to diploid, isolated monosomy 22 and complex iFISH karyotypes.

Among other markers, the CD55 (decay-accelerating factor; DAF) complement regulatory protein showed a uniquely high expression among diploid *vs.* cytogenetically altered meningiomas. Increased expression of complement regulatory proteins by tumor cells has been associated with resistance to complement-mediated cytotoxicity in several subtypes of solid tumors [472, 473]; indeed, increased expression of CD55 mRNA has been proposed as a mechanism that facilitates tumor survival, leading to a more aggressive tumor behavior and a poorer patient outcome [474]. Although CD55 did not show a significant association with RFS and more aggressive features of the disease, an inverse correlation was found between cell surface CD55 protein and total mRNA levels in meningiomas (with greater mRNA expression among cytogenetically complex tumors). Such apparent discrepancy between cell surface protein levels and mRNA expression may be due to an altered balance between protein synthesis, degradation, secretion and/or mobilization of stored CD55. In this regard, it should be noted that recent reports demonstrate the presence of soluble CD55 (sCD55) at the extracellular matrix level in several different tumor types [474].

Similarly to diploid tumors, meningiomas with isolated monosomy 22/del(22q) also showed a unique protein (and mRNA) expression profile *vs.* other cytogenetic subgroups of meningiomas, such profile consisting of significantly higher levels of Cybcl2 and PDGFRβ. These findings point out the potential relevance of PDGFRβ in this subgroup of meningiomas [175, 475], where it may be associated with inhibition of apoptosis [310, 423, 476] through activation of Akt [477, 478]. Finally, compared to other meningiomas, cytogenetically complex tumors showed uniquely low protein and mRNA expression levels of the CD13 ectoenzyme (aminopeptidase N; APN), in addition to a higher proliferation index. CD13 has been involved in a variety of cellular functions, including the control of tumor cell proliferation and invasion [414], and its relevance in meningiomas has already been reported by others. In this regard, our results are in line with those reported by Mawrin et al. [419] describing a significant reduction of APN (CD13) mRNA and protein expression levels, as well as its enzymatic activity,

in high-grade meningiomas; as previously suggested, unbalanced expression of APN (CD13) and SPARC might favor meningioma invasion [419, 479]. Moreover, cytogenetically complex meningiomas also showed decreased expression of the CD9 and CD81 tetraspanins, both of which have been involved in the regulation of cell morphology, motility, invasion, fusion and signaling, in a variety of normal tissues and several different cancer types [480]. In this regard, growing evidence indicates that decreased expression of several members of the tetraspanin family of adhesion molecules are associated with malignant progression of solid tumors, and both CD9 [417, 418] and CD81 [415] have been considered markers of malignancy, as lack of expression of CD9 and CD81 has been associated with lower integrin-dependent adhesion and enhanced cell growth [481]. These observations may contribute to explain, at least in part, the increased proliferation index of tumor cells from cytogenetically complex *vs.* other meningiomas. Finally, expression of the HER2/neu growth factor receptor protein was also significantly decreased among tumors with a complex karyotype *vs.* other meningiomas. Of note, expression of Her2/neu should be carefully evaluated for appropriate interpretation since this protein is expressed at the cytoplasmic membrane, but as other ErbB receptors (e.g. EGFR) it may undergo internalization and/or cleavage due to recycling of the receptor between the plasma membrane and the endosomal compartments, and because of protease-mediated cell surface cleavage in activated cells, respectively [411, 482]. This might contribute to explain why despite lower protein levels were found in cytogenetically complex *vs.* other meningiomas, no differences were detected at the mRNA level between the distinct cytogenetic tumor subtypes. Therefore, lower Her2/neu protein levels on the cell membrane of cytogenetically complex meningiomas may potentially reflect a higher cell activation and protein processing. Overall, these findings support previous observations [483-485] suggesting the relevance of ErbB receptors in the biology of meningiomas.

The level of expression of distinct proteins on tumor cells was also associated with other features of the disease, including patient outcome. Of note, higher expression of both CD44 and CD53 was associated with a shorter RFS, in addition to tumor grade and cytogenetics. Interestingly, CD44 is involved in the regulation of cell-cell and cell-matrix adhesion, acting as a functional antagonist of the merlin protein [92, 97], which is the product of the NF2 tumor suppressor gene that is frequently lost during meningioma tumorigenesis [3, 92, 118]. In this regard, it has been described that monosomy 22 is closely associated with the presence of coding *NF2* mutations [87, 106], although not all cases with monosomy 22 carry *NF2* mutations [87]. Even more, greater CD44 expression in higher grade meningiomas has also been previously found by others [59, 421], which would also support our findings. Regarding CD53, to the best of our knowledge this is the first report in which this protein is specifically

investigated in meningiomas; however, as a member of the tetraspanin family, CD53 has been associated with cell adhesion and motility [480, 486] and, similarly to other tetraspanin family members (e.g. CD9/CD81), it might be related to tumor invasion [415, 417, 418, 481].

Although expression of bcl2 was not associated with patient outcome, it was expressed at higher levels in female tumors, with a convexity/parasagittal localization and a fibroblastic histopathology. For decades now, the role of bcl2 in meningiomas has been extensively investigated; however, although in other studies bcl2 levels have been associated with the WHO tumor grade and prognosis, these observations could not be confirmed by others. Based on our results, it may be speculated that such discrepancies could be due to the different levels at which bcl2 is expressed in the different histological subtypes of grade I meningiomas, and potentially also to its greater levels among tumors with isolated monosomy 22/del(22q).

Other clinical-phenotypic associations observed in our study included higher expression of CD63 in convexity/parasagittal tumors, lower levels of IGFR in spinal tumors and greater expression of CD99 in transitional *vs.* meningothelial meningiomas, together with higher CD38 levels among cases with moderate to severe edema. IGF has long been shown to regulate, at least in part, meningioma growth through its effect on tumor cell proliferation and survival [426, 487]; however, to the best of our knowledge its expression has not been previously related in meningiomas to other disease features. Regarding CD99, this marker is used in the histological diagnostic work-up of some CNS tumors [488, 489], being positive in spindle cell tumors (e.g. meningeal hemangiopericytoma and solitary fibrous tumors of the meninges) [490]; among our cases, CD99 also showed higher levels of expression among those histopathological subtypes of meningiomas which show spindle-shaped cells (fibroblastic/transitional). Of note, CD38 was the only immunophenotypic marker whose expression was significantly related with brain edema. CD38 is a multifunctional ectoenzyme essential for the regulation of intracellular calcium. Brain edema in meningioma patients is mainly due to an increase in the permeability of the blood-brain barrier (BBB) [491]. Since regulation of extracellular and intracellular calcium levels seems to be critical in the normal functioning of the BBB [492], and calcium overload is a main cause of ischemia and brain edema after trauma [493], our results suggest that CD38 expression by tumor cells could play a role in the genesis of edema in these patients.

Interestingly, several recent studies have focused on the mutational status of several genes other than *NF2*, which seem to be important for the biology of meningiomas; these include mutations of genes such as *AKT1*, a constituent of the PI3K which has been related to specific localizations and histological subtypes of meningiomas, and *KLF4*, a gene typically

altered in secretory meningiomas [106, 144]. In our series, PDGFRβ expression (a gene also related to the PI3K pathway) was associated with meningioma histology and cytogenetics.

In summary, here we show that the protein expression profile of individual meningioma cells, as evaluated by MFC immunophenotyping, is closely associated with tumor cytogenetics, which may reflect the involvement of different signaling pathways in the distinct cytogenetic subgroups of meningiomas. In addition, our data also show a close association between some of the markers investigated (e.g. CD44 and CD53) and patient RFS, suggesting that specific protein expression profiles may translate into a more aggressive *vs.* mild clinical behavior of the tumor. Further investigations in larger series of patients, analyzed with extended antibody panels and the flow cytometry techniques here described, are required to confirm this hypothesis.

Chapter VII

Proposal for a new risk stratification classification for meningioma based on patient age, WHO tumor grade, size, localization, and karyotype

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1. Abstract

Tumor recurrence remains the major clinical complication of meningiomas, the majority of recurrences occurring among WHO grade I/benign tumors. In the present study, we propose a new scoring system for the prognostic stratification of meningioma patients based on the analysis of a large series of 302 meningiomas followed for a median of >5 years, in which tumor cytogenetics was systematically investigated by interphase fluorescence *in situ* hybridization and further validated in an independent series (n=132) by high-density (500K) single nucleotide polymorphism (SNP)-arrays. Overall, our results showed an adverse impact on patient RFS for males, presence of brain edema, younger patients (<55 years), tumor size >50mm, tumor localization at intraventricular and anterior cranial base areas, WHO grade II/III meningiomas and complex karyotypes, the latter five variables showing an independent predictive value in multivariate analysis. Based on these parameters, a prognostic score was established for each individual case and patients were stratified into 4 risk-categories with a significantly different ($p<0.001$) outcome; this included a good-prognosis group consisting of ≈20% of cases which showed a RFS of 100%±0% at 10 years and a very poor-prognosis group with a RFS rate of 0%±0% at 10 years. The prognostic impact of the scoring system here proposed was also retained when WHO grade I cases were separately considered ($p<0.001$). In summary, these results indicate that based on this risk-stratification classification, different strategies may be adopted for the follow-up, and eventually also for the treatment, of meningioma patients, at different risk of relapse.

2. Introduction

Meningiomas are usually considered to be slow-growing clinically benign tumors, which can be cured by conventional surgical procedures [4, 5, 8]. However, between 10% and 30% of those cases who had undergone complete tumor resection, and around 60% of tumors who underwent subtotal tumor resection, show tumor recurrence at 10 years, in association with a significantly poorer overall survival [32, 41]. So far, multiple different independent prognostic factors have been identified in meningiomas and some prognostic scoring systems have been proposed [79, 223, 494-496] to predict the outcome of individual patients already at diagnosis [8]. Among such prognostic factors, tumor cytogenetics together with the WHO tumor grade, the extent of tumor resection, patient age and tumor localization have proven to be particularly informative [32, 60, 76, 497, 498].

Despite risk stratification based on individual prognostic factors and some combinations of them [45, 61, 494], has proven to contribute to predict patient outcome, they have not been fully adopted in routine clinical practice. Among other reasons, this relates to the fact that both the individual risk factors and the prognostic scores that have been proposed so far, still fail to predict the outcome of a significant proportion of cases included both in the low- and the high-risk patient categories. As an example, whereas atypical and anaplastic tumors show a greater recurrence rate than WHO grade I meningiomas, the majority of meningioma relapses (around 80% of all recurrences) still occur among WHO grade I cases [60]. Similarly, with the Maillo et al. [45] score built on the basis on chromosome 14 abnormalities, patient age, and tumor histopathology, a significant number of recurrences are still observed among the good-prognosis category, particularly within WHO grade I/benign meningiomas (e.g. 12% of all relapses), at the same time a significant fraction of the high-risk cases (e.g. around 50% of high-risk WHO grade I tumors) are relapse-free long-term survivors. Based on all the above, there is an urgent need for a more reliable risk stratification classification of meningiomas, for adequate definition of the most efficient follow-up strategies and the potential adoption of different treatment approaches in an individual patient basis.

Here we analyzed a series of 302 meningiomas with a median follow-up of more than 5 years for the most relevant clinical and biological features of the disease, including tumor cytogenetics as assessed by iFISH. Our major goal was to identify a combination of prognostic factors that could be used to stratify meningioma patients according to their risk of recurrence; such risk stratification should therefore allow the identification of a good-prognosis group of patients who do not require follow-up and a poor prognosis group for whom closer monitoring,

including potential adoption of additional treatment measures, would be required for early diagnosis and/or prevention of tumor recurrence, particularly among histologically benign/grade I meningiomas. An additional group of 132 cases studied by single nucleotide polymorphism (SNP)-arrays was included in this study for validation purposes.

3. Results

3.1. Cytogenetic profile of meningiomas

From the 302 meningiomas analyzed, 90 (30%) displayed no cytogenetic alterations by iFISH for the 11 chromosomes analyzed. In contrast, the other 212 (70%) cases showed numerical alterations for ≥1 chromosome. As expected, chromosome 22 was the most frequently altered chromosome (173/302 cases; 57%), its alteration mainly consisting of monosomy 22/22q deletions (166/173 altered cases). Other recurrently altered chromosomes included chromosome Y in males (26/91 cases; 28%), chromosome 1p (67/302 cases; 22%), chromosome 14 (47/302; 16%), chromosome X in females (28/211; 13%), chromosome 1q (37/302; 12%) and chromosome 10 (31/302; 10%). Overall, chromosomal losses were more frequently observed than gains (55% *vs.* 2% for chromosome 22, 26% *vs.* 2% for chromosome Y, 21% *vs.* 1% for chromosome 1p, 13% *vs.* 3% for chromosome 14, 12% *vs.* 1% for chromosome X and 6% *vs.* 4% for chromosome 10), except for chromosome 1q which was more frequently gained (10%) than lost (2%). All other chromosomes analyzed were found to be altered at lower (≤8%) frequencies (Supplementary Table 3).

Around half of all cytogenetically altered cases (n=106; 35%) showed isolated alterations of a single chromosome, whereas the other half (n=106; 35%) displayed cytogenetic alterations (losses and/or gains) involving ≥2 chromosomes (Table 12). Among those cases carrying isolated chromosomal alterations, the most frequent pattern consisted of loss of one chromosome 22 or del(22q) (83/106; 78%), while isolated involvement of other chromosomes was restricted to a few cases: isolated loss of chromosome 1p was found in 9 tumors, loss of chromosome Y in 4, loss of chromosome 10 in 2 and losses of chromosomes X and 9 were found in one case each; isolated gains of chromosomes 1q and 14 were detected in 5 and 1 patients, respectively.

Except for chromosome 10 in the whole series, and chromosomes Y and X in males, alterations of all other individual chromosomes were significantly more frequent among WHO grade II/III *vs.* grade I tumors (p≤0.001; Supplementary Table 3). In line with these findings, diploid tumors and cases with single chromosomal alterations were more frequently (p≤0.001) observed among grade I meningiomas - 82/90 (91%) diploid cases and 101/106 (95%) tumors with only one altered chromosome corresponded to WHO grade I tumors -, while more complex cytogenetic patterns predominated among grade II/III meningiomas - 25/38 (66%) grade II/III tumors showed a complex karyotype -. Of note, tumors with complex karyotypes represented more than half of the male patients (51/91; 56%), whereas they only accounted for 26% of female cases (55/211; p≤0.001).

3.2. Prognostic impact of tumor cytogenetics and other relevant clinical and histopathological features of the disease

Tumor cytogenetics as assessed by iFISH showed a significant association with the incidence of relapses and patients' RFS (Supplementary Table 3). Accordingly, alterations of chromosomes 1p, 1q, 7, 9, 10, 14, 18 and 22 in the whole series, and of chromosome X in females, were associated with both a higher incidence of relapses ($p<0.05$; Supplementary Table 3), and/or a significantly shorter RFS ($p<0.03$; Supplementary Table 3 and Supplementary Figure 2). Multivariate analysis including all individual chromosomes with a significant impact on RFS in the univariate study, showed that only the alteration of chromosome 14 ($p=0.001$), gains of chromosome 7 ($p=0.047$) and losses of chromosome 18 ($p<0.001$) had an independent predictive value for a poorer outcome (Supplementary Table 3). In turn, when the three major iFISH cytogenetic subgroups of meningiomas were considered, patients with tumors carrying a complex karyotype showed a significantly shorter RFS than cases showing a diploid karyotype and presence of isolated chromosomal alterations (75% RFS of 78 months *vs.* not reached, respectively; $p=0.001$) (Table 12; Figure 27H); of note, individual chromosomes lost their independent prognostic value once these three cytogenetic profiles were included in the analysis (data not shown). Patients carrying tumors with complex karyotypes showed lower 5-, 10- and 15-years RFS rates than cases with diploid karyotypes or isolated chromosomal alterations (79%±5% *vs.* 94%±3% and 91%±3%, 63%±7% *vs.* 90%±6% and 88%±4% and, 55%±8% *vs.* 78%±12% and 80%±7%, respectively) (Table 12). Of note, within those cases with an isolated chromosomal alteration, patients who had isolated monosomy 22/del(22q) showed a slightly better RFS than patients with isolated alterations of other chromosomes - 75% RFS not reached *vs.* 82 months ($p>0.05$); 5-, 10- and 15-years RFS rates of 92%±4% *vs.* 89%±8%, 92%±4% *vs.* 74%±15% and 82%±8% *vs.* 74%±15%, respectively (Table 12) -. Of note, combined assessment of chromosomes 1p, 7, 14, 18 and 22 represented the minimal chromosomal panel that could be applied for the identification of the three most common subtypes of genetic patterns as used in the prognostic scoring system.

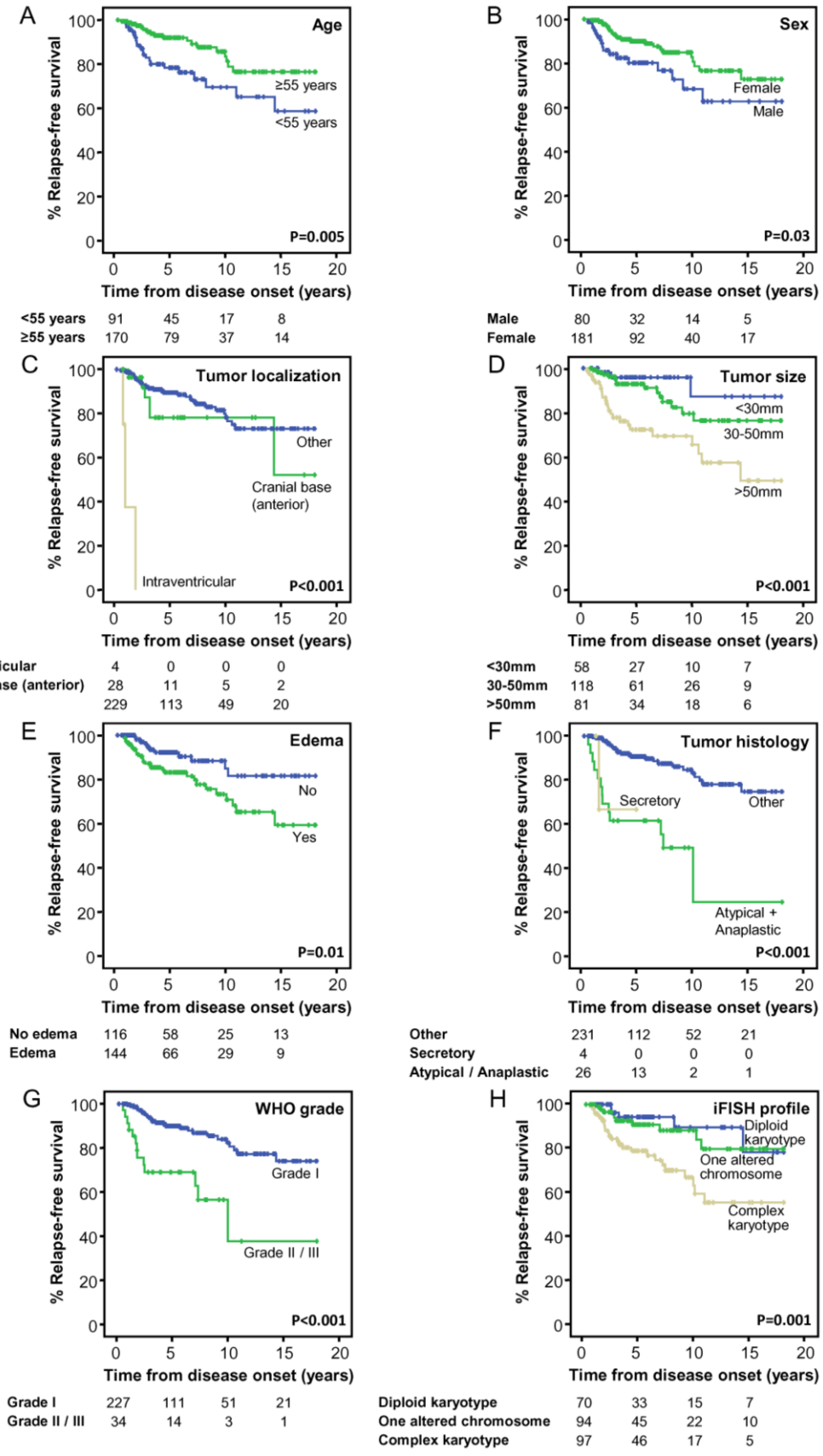
Table 12. Clinical and biological characteristics of the meningioma patients included in this study (n=302) and their association with disease outcome in those 261 cases with available follow-up data.

Variables		Patient distribution	No. of recurrences	P-value	% of patients relapse-free			75% RFS (months)	Univariate analysis	Multivariate analysis #	
					5y-RFS *	10y-RFS *	15y-RFS *		P-value	P-value	HR (95% CI)
Age	<55 years	97 (32%)	23 (24%)	0.001	78% ± 5%	70% ± 7%	59% ± 9%	85	0.005	0.01	3 (1-5)
	≥55 years	205 (68%)	19 (9%)		92% ± 2%	83% ± 4%	77% ± 6%	NR			
Sex	Male	91 (30%)	18 (20%)	0.04	80% ± 5%	68% ± 8%	63% ± 9%	98	0.02		
	Female	211 (70%)	24 (11%)		90% ± 3%	83% ± 4%	73% ± 6%	172			
Cytogenetic profile	Diploid karyotype	90 (30%)	5 (6%)	<0.001	94% ± 3%	90% ± 6%	78% ± 12%	NR	0.001	0.002	
	One altered chromosome	106 (35%)	10 (9%)		91% ± 3%	88% ± 4%	80% ± 7%	NR			
	Monosomy22/del(22q)	83 (27%)	7 (8%)		92% ± 4%	92% ± 4%	82% ± 8%	NR			7 (1-33)
	Other	23 (8%)	3 (13%)		89% ± 8%	74% ± 15%	74% ± 15%	82			15 (4-64)
	Complex karyotype	106 (35%)	27 (25%)		79% ± 5%	63% ± 7%	55% ± 8%	78			17 (3-113)
WHO grade	Grade I	264 (87%)	28 (11%)	<0.001	90% ± 2%	82% ± 4%	74% ± 5%	172	<0.001	<0.001	
	Grade II	30 (10%)	12 (40%)		64% ± 10%	56% ± 11%	37% ± 17%	22			7 (3-17)
	Grade III	8 (3%)	2 (25%)		86% ± 13%	-	-	85			6 (1-30)
Tumor localization	Convexity	63 (21%)	10 (16%)	N.S.	90% ± 5%	76% ± 8%	64% ± 11%	122	<0.001	<0.001	
	Parasagittal	56 (18%)	7 (13%)		91% ± 5%	85% ± 7%	78% ± 9%	237			
	Falcine	35 (12%)	3 (9%)		89% ± 6%	89% ± 6%	89% ± 6%	NR			
	Cranial base (anterior)	38 (13%)	6 (16%)		78% ± 9%	78% ± 9%	52% ± 22%	172			17 (4-75)
	Cranial base (middle)	47 (15%)	6 (13%)		93% ± 5%	76% ± 10%	68% ± 12%	127			
	Cranial base (posterior)	22 (7%)	3 (14%)		82% ± 10%	82% ± 10%	82% ± 10%	NR			
	Tentorial	11 (3%)	2 (18%)		78% ± 14%	78% ± 14%	-	NR			
	Intraventricular	4 (2%)	3 (75%)		0%	-	-	9			17 (4-84)
	Spinal	26 (9%)	2 (8%)		96% ± 4%	77% ± 18%	77% ± 18%	NR			
Tumor histology	Meningothelial	147 (49%)	18 (12%)	<0.001	90% ± 3%	83% ± 4%	75% ± 6%	172	<0.001		
	Fibroblastic	17 (5%)	2 (12%)		92% ± 8%	0%	0%	82			
	Transitional	57 (19%)	6 (11%)		89% ± 6%	74% ± 14%	59% ± 17%	109			
	Psammomatous	34 (11%)	1 (3%)		95% ± 5%	95% ± 5%	95% ± 5%	NR			
	Angioblastic	7 (2%)	0 (0)		100%	100%	-	NR			
	Secretory	5 (2%)	1 (20%)		67% ± 27%	-	-	19			

Table 12. *(continued)*

	Atypical	24 (8%)	12 (50%)		57% ± 11%	49% ± 12%	25% ± 18%	21			
	Chordoid	3 (1%)	0 (0)		-	-	-	NR			
	Anaplastic	5 (2%)	2 (40%)		80% ± 18%	40% ± 30%	-	85			
	Rhabdoid	2 (1%)	0 (0)		-	-	-	NR			
	Papillary	1 (0)	0 (0)		-	-	-	NR			
Edema	No	128 (43%)	11 (9%)	0.03	92% ± 3%	85% ± 5%	82% ± 6%	NR	0.01		
	Yes	173 (57%)	31 (18%)		83% ± 3%	73% ± 5%	59% ± 8%	109			
Tumor size	<30 mm	66 (22%)	3 (5%)		96% ± 3%	87% ± 9%	87% ± 9%	NR			
	30-50 mm	139 (47%)	15 (11%)	<0.001	93% ± 3%	79% ± 6%	76% ± 6%	237	<0.001	0.001	
	>50 mm	92 (31%)	24 (26%)		72% ± 6%	69% ± 6%	49% ± 10%	51			5 (1-21)

Results expressed as number of cases and percentage between brackets or as * percentage of cases ± SE (standard error); NR: 75% RFS not reached; HR: Hazard ratio; CI: confidence interval; # the category with the best prognosis was selected as reference group.


A
Age
≥55 years
<55 years
P=0.005
% Relapse-free survival
Time from disease onset (years)
<55 years 91 45 17 8
≥55 years 170 79 37 14
B
Sex
Female
Male
P=0.03
Male 80 32 14 5
Female 181 92 40 17
C
Tumor localization
Other
Cranial base (anterior)
Intraventricular
P<0.001
Intraventricular 4 0 0 0
Cranial base (anterior) 28 11 5 2
Other 229 113 49 20
D
Tumor size
<30mm
30-50mm
>50mm
P<0.001
<30mm 58 27 10 7
30-50mm 118 61 26 9
>50mm 81 34 18 6
E
Edema
No
Yes
P=0.01
No edema 116 58 25 13
Edema 144 66 29 9
F
Tumor histology
Secretory
Other
Atypical + Anaplastic
P<0.001
Other 231 112 52 21
Secretory 4 0 0 0
Atypical / Anaplastic 26 13 2 1
G
WHO grade
Grade I
Grade II / III
P<0.001
Grade I 227 111 51 21
Grade II / III 34 14 3 1
H
iFISH profile
Diploid karyotype
One altered chromosome
Complex karyotype
P=0.001
Diploid karyotype 70 33 15 7
One altered chromosome 94 45 22 10
Complex karyotype 97 46 17 5

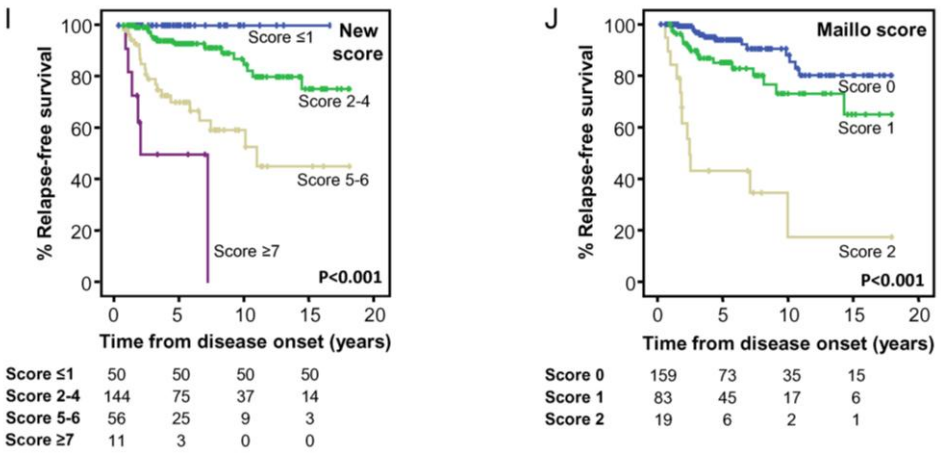


Figure 27. Clinical, biological and genetic features of meningiomas which showed a significant impact on patient relapse-free survival (RFS; n=261). Relapse-free survival curves of meningioma patients according to patient age (panel A) and gender (panel B), tumor localization (panel C) and size (panel D), presence *vs.* absence of edema (panel E), tumor histology (panel F), WHO grade (panel G) and its iFISH cytogenetic profile (panel H). In the last two panels (panels I and J), patients' RFS curves according to the new prognostic scoring system here proposed (panel I) and that previously reported by Maillo *et al.* (J Clin Oncol, 2003), are shown.

In addition to tumor cytogenetics, several other clinical and histopathological features of the disease also showed an adverse impact on patients' RFS, namely a younger age (<55 years; p=0.005), male gender (p=0.02), WHO grade II/III (p<0.001), atypical or anaplastic tumor histopathology (p<0.001), intraventricular or anterior cranial base tumor localization (p<0.001), tumor size >50 mm (p<0.001) and presence of brain edema (p=0.01) (Table 12 and Figures 27A-G). Multivariate analysis of prognostic factors showed that tumor cytogenetics (p=0.002), together with the WHO tumor grade (p<0.001), localization (p<0.001) and size (p=0.001), as well as patient age (p=0.01), represented the best combination of independent prognostic factors for predicting RFS of meningioma patients (Table 12). Of note, the Simpson grade of resection did not show a significant (p>0.05) impact on patient RFS.

Based on the above five variables (patient age, tumor cytogenetics, WHO grade, localization and size), a prognostic score was established for each individual patient, using the criteria described in Table 13. Once this score was applied, patients were divided into four risk groups: (i) low-risk patients with a score ≤1 (n=62), (ii) intermediate-1 cases with a score of between 2 and 4 (n=170), (iii) intermediate-2 cases with a score of between 5 and 6 (n=59), and; (iv) high-risk cases with a score ≥7 (n=11). Patients included in these four risk categories showed progressively shorter 75% RFS rates (p<0.001) from low (not reached), to int-1 (not reached), int-2 (38 months) and high-risk cases (15 months). Similarly, progressively lower 5-,

10- and 15-years RFS rates were also found for low (100%±0%), int-1 (93%±2%, 85%±5% and 75%±7%, respectively), int-2 (70%±7%, 59%±16% and 45%±11%, respectively) and high-risk cases (50%±16%, 0%±0% and 0%±0%, respectively) (Table 13; Figure 27I). For comparison purposes, RFS curves for the same series of cases, as defined according to the scoring system previously proposed by Maillo *et al.* [45], are shown in Figure 27J.

Table 13. Scoring criteria used for those five variables included in the new prognostic scoring system proposed in this study.

Score	Age (years)	WHO grade	Cytogenetic profile	Tumor size (mm)	Tumor localization
0	≥55	I	Diploid karyotype	<30	Other
1	<55	II	Monosomy 22/del(22q)	30-50	Cranial base (anterior)
2		III	One altered chromosome (other than Chr22)	>50	Intraventricular
3			Complex karyotype		

Risk group (overall score)	Patient distribution	N. of recurrences	p-value	% of patient relapse-free			75% RFS (months)	p-value
				5y-RFS *	10y-RFS *	15y-RFS *		
	All WHO grade tumors							
Low (0-1)	62 (21%)	0 (0%)	<0.001	100% ± 0%	100% ± 0%	100% ± 0%	NR	<0.001
Intermediate-1 (2-4)	170 (56%)	15 (9%)		93% ± 2%	85% ± 5%	75% ± 7%	NR	
Intermediate-2 (5-6)	59 (20%)	21 (36%)		70% ± 7%	59% ± 8%	45% ± 11%	38	
High (≥7)	11 (4%)	6 (55%)		50% ± 16%	0% ± 0%	0% ± 0%	15	
	WHO grade I tumors							
Low (0-1)	59 (22%)	0 (0%)	<0.001	100% ± 0%	100% ± 0%	100% ± 0%	NR	<0.001
Intermediate-1 (2-4)	163 (62%)	14 (9%)		94% ± 2%	85% ± 5%	76% ± 7%	NR	
Intermediate-2 (5-6)	40 (15%)	12 (30%)		72% ± 8%	61% ± 10%	49% ± 15%	51	
High (≥7)	2 (1%)	2 (100%)		0% ± 0%	0% ± 0%	0% ± 0%	15	

Results expressed as number of cases and percentage between brackets or as *percentage of cases ± SE (standard error); NR: 75% RFS not reached.

Table 14. Clinical and biological characteristics of WHO grade I meningiomas (n=264) and their association with disease outcome in 227 cases with available follow-up data.

Variables		Patient distribution	No. of recurrences	P-value	% of patients relapse-free			75% RFS (months)	Univariate analysis	Multivariate analysis #	
					5y-RFS *	10y-RFS *	15y-RFS *			P-value	HR (95% CI)
Age	<55 years	81 (31%)	15 (19%)	0.009	83% ± 5%	76% ± 7%	63% ± 10%	130	0.02	0.007	3 (1-8)
	≥55 years	183 (69%)	13 (7%)		94% ± 2%	86% ± 4%	81% ± 5%	NR			
Sex	Male	72 (27%)	10 (14%)	NS	87% ± 5%	73% ± 9%	66% ± 10%	109	NS		
	Female	192 (73%)	18 (9%)		91% ± 3%	86% ± 4%	77% ± 6%	NR			
Cytogenetic profile	Diploid karyotype	82 (31%)	4 (5%)	0.01	96% ± 3%	91% ± 6%	79% ± 12%	NR	0.01	<0.001	
	One altered chromosome	101 (38%)	8 (8%)		93% ± 3%	90% ± 4%	82% ± 7%	NR			
	Monosomy22/del(22q)	81 (31%)	6 (7%)		93% ± 3%	93% ± 3%	83% ± 8%	NR			12 (2-68)
	Other	20 (7%)	2 (10%)		93% ± 6%	78% ± 15%	78% ± 15%	NR			31 (6-160)
	Complex Karyotype	81 (31%)	16 (20%)		82% ± 5%	67% ± 8%	62% ± 9%	109			32 (3-296)
Tumor localization	Convexity	55 (21%)	7 (13%)	NS	90% ± 5%	85% ± 7%	70% ± 11%	131	<0.001	0.002	
	Parasagittal	44 (17%)	3 (7%)		94% ± 4%	86% ± 9%	86% ± 9%	NR			
	Falcine	29 (11%)	1 (3%)		95% ± 5%	95% ± 5%	95% ± 5%	NR			
	Cranial base (anterior)	37 (14%)	6 (16%)		78% ± 9%	78% ± 9%	52% ± 22%	172			21 (5-99)
	Cranial base (middle)	43 (16%)	5 (12%)		96% ± 4%	78% ± 10%	70% ± 12%	127			
	Cranial base (posterior)	21 (8%)	2 (10%)		87% ± 9%	87% ± 9%	87% ± 9%	NR			
	Tentorial	11 (4%)	2 (18%)		78% ± 14%	78% ± 14%	78% ± 14%	NR			
	Intraventricular	2 (1%)	1 (50%)		0%	-	-	23			11 (1-113)
	Spinal	22 (8%)	1 (5%)		80% ± 18%	80% ± 18%	80% ± 18%	NR			
Tumor histology	Meningothelial	147 (56%)	18 (12%)	NS	90% ± 3%	82% ± 4%	75% ± 6%	172	NS		
	Fibroblastic	17 (6%)	2 (12%)		92% ± 8%	-	-	82			
	Transitional	57 (22%)	6 (11%)		89% ± 6%	74% ± 14%	59% ± 17%	109			
	Psammomatous	34 (13%)	1 (3%)		95% ± 5%	95% ± 5%	95% ± 5%	NR			
	Angioblastic	4 (1%)	0 (0%)		-	-	-	NR			
	Secretory	5 (2%)	1 (20%)		67% ± 27%	-	-	19			
Edema	No	120 (46%)	9 (8%)	NS	94% ± 3%	86% ± 5%	83% ± 6%	NR	NS		
	Yes	143 (54%)	19 (13%)		86% ± 4%	79% ± 5%	65% ± 9%	131			
Tumor size	<30 mm	63 (24%)	3 (5%)	0.001	96% ± 3%	87% ± 9%	87% ± 9%	NR	0.002	0.03	
	30-50 mm	117 (45%)	8 (7%)		96% ± 2%	85% ± 6%	81% ± 7%	NR			
	>50 mm	79 (31%)	17 (22%)		77% ± 6%	73% ± 6%	55% ± 11%	78			4 (1-20)

Results expressed as number of cases and percentage between brackets or as *percentage of cases ± SE (standard error); NS: statistically not significant (p>0.05); NR: 75% RFS not reached. HR: Hazard ratio; CI: confidence interval; # the category with the best prognosis was selected as reference group.

Most interestingly, when WHO grade I tumors were exclusively considered, tumor cytogenetics retained its prognostic significance (p=0.01; Table 14; Figure 28D), together with patient age, tumor localization and size. Thus, complex karyotypes, age <55 years, intraventricular and anterior cranial base localization and a tumor size ≥50mm, were all associated with shorter 75% RFS rates both in the univariate (p=0.01, p=0.02, p<0.001 and p=0.002, respectively; Table 14; Figure 28A-C) and in the multivariate analyses (p<0.001, p=0.007, p=0.002 and p=0.03, respectively; Table 14). When the proposed score was specifically adjusted for WHO grade I tumors (Table 13), the significantly different outcome of low-, int-1, int-2 and high-risk cases was retained, with 75% RFS rates of not reached, not reached, 51 and 15 months, respectively (p<0.001; Table 13; Figure 28E). This clearly improved the prediction obtained with the previously proposed Maillo *et al.* score particularly among the low-risk group (Figure 28F).

3.3. Validation of the iFISH profiles by high-density copy number arrays.

To validate the iFISH cytogenetic profiles, the prognostic value of the copy number (CN) profiles obtained for the 24 human chromosomes by SNP-arrays were evaluated in an additional group of 132 cases pooled, from our institution (n=50)[118] and another series from the literature (n=82) with publicly available data. Based on SNP-arrays, 41 (31%) of these 132 cases showed no CN alterations for any of the 24 chromosomes evaluated, 40 tumors (30%) showed isolated alterations of a single chromosome, including monosomy 22/del(22q) in 35/40 cases, and 51 patients (39%) displayed complex karyotypes. Once again, the majority of WHO grade II/III tumors showed a CN pattern compatible with a complex karyotype (27/34; 79%), whereas low grade meningiomas most frequently had a diploid karyotype or isolated alterations of a single chromosome (74/98 cases; 76%). In addition, meningioma patients carrying complex karyotypes associated with CN alterations of ≥2 chromosomes also showed a poorer outcome than cases with a diploid karyotype or isolated chromosomal alterations (p=0.005; Figure 29A); similarly, we could also confirm in this validation series, the improved predictive value of the new prognostic scoring system defined above (Figure 29B) over the Maillo *et al.* score (Figure 29C), particularly for the low-risk category.

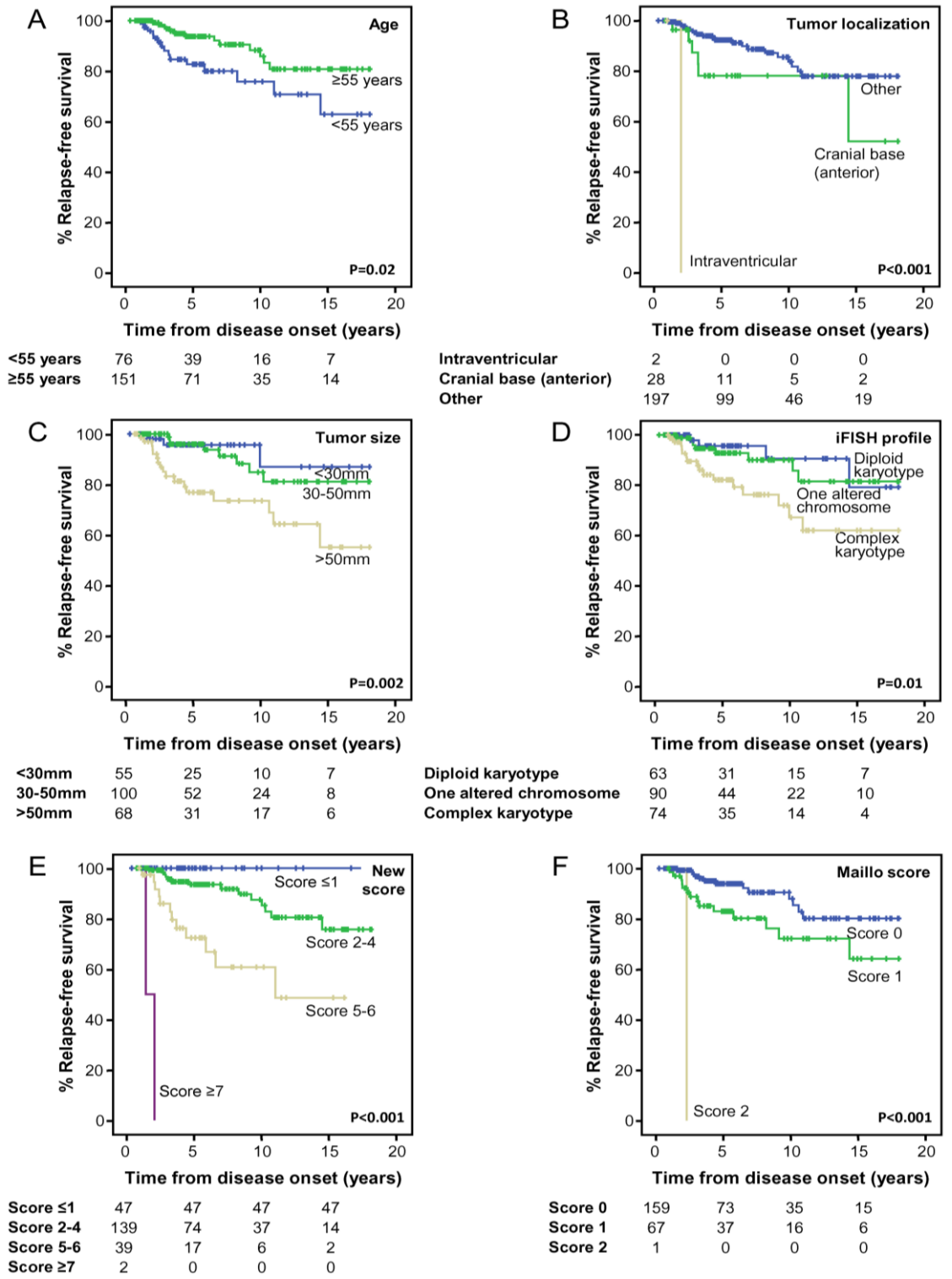


Figure 28. Clinical, biological and genetic features of WHO grade I meningiomas with a significant impact on patient relapse-free survival (RFS; n= 227). Relapse-free survival curves of WHO grade I meningioma patients grouped according to patient age (panel A), tumor localization (panel B), size (panel C) and its iFISH cytogenetic profile (panel D). In panels E and F, RFS curves for the same cases grouped according to the new prognostic scoring system proposed here and that previously reported by Maillo *et al.* (J Clin Oncol, 2003) are shown, respectively.

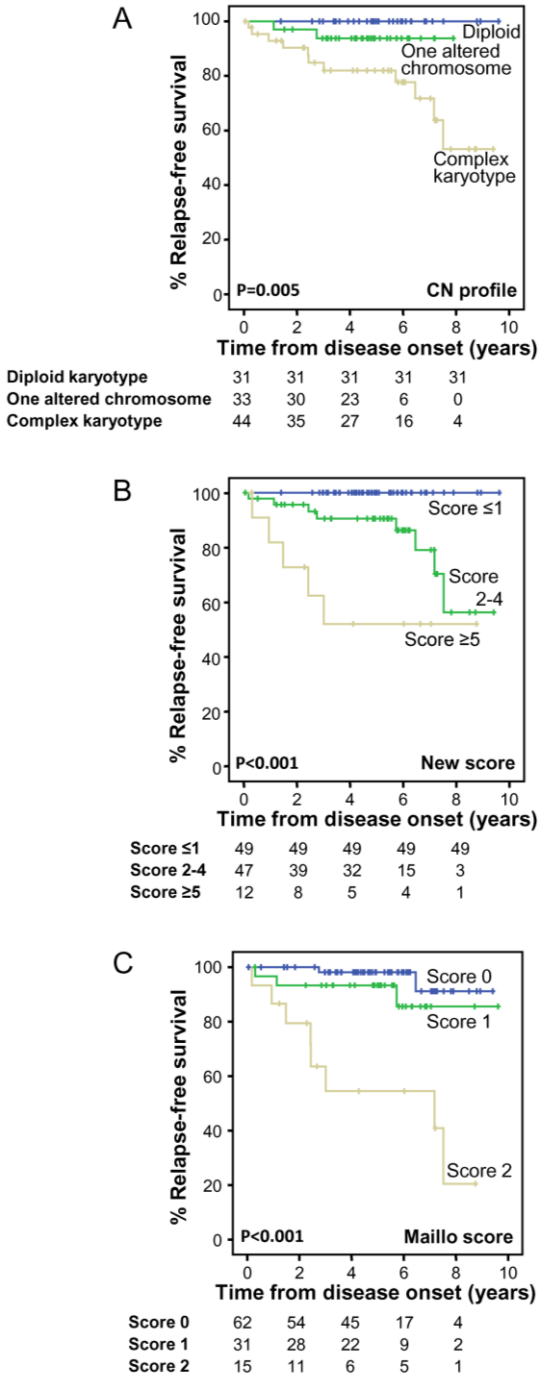


Figure 29. Impact of tumor karyotype as defined by the chromosomal copy number profile as analyzed by SNP-arrays, on relapse-free survival of meningioma patients. Relapse-free survival curves of meningioma patients classified according to the CN patterns (diploid, isolated alteration of a single chromosome and complex karyotypes), for the two series of cases analyzed by SNP-arrays (n=132) which have been published so far in the literature, are shown in panel A; only cases with follow-up data (n=108) were considered for RFS analysis. In panels B and C, RFS curves of the same cases grouped according to the new prognostic scoring system here proposed (panel B; adjusted for the information available for the Lee series, which did not include data on tumor localization and size) and that previously reported by Maillo *et al.* (J Clin Oncol, 2003) (panel C), based on chromosome copy number data obtained by SNP-arrays, are shown.

4. Discussion

Tumor recurrence remains the major clinical complication of meningioma [8, 32]. Although multiple prognostic factors have long been identified [8, 32, 41, 44, 499] and some prognostic classifications, which include tumor cytogenetics, have been proposed [62, 223, 495], prediction of tumor recurrence in an individual patient basis still remains a challenge [8, 32]. Because of this, a relatively uniform close follow-up of every newly-diagnosed patient has been adopted in most centers. In fact, a significant percentage (12% to >50%) of all recurrences still occur among the good-prognosis patient categories, at the same time a substantial fraction of all high-risk cases are relapse-free long-term survivors [8, 32, 60]. Altogether, this points out the need for improved prognostic stratification systems based on the analysis of large numbers of cases followed for long periods of time, in which information about all potentially relevant prognostic parameters is assessed at diagnosis.

In the present study, we analyzed a large series of meningiomas with a uniform median follow-up of >5 years, in which the most relevant clinico-biological, histopathological and cytogenetic features of the disease had been systematically investigated at diagnosis. Our major goal was to identify a combination of prognostic factors that could stratify meningioma patients into different risk categories; such risk categories should include on one side, very low-risk cases which do not require follow-up and, on the other side, a poor-prognosis category where additional/alternative therapeutic measures might be required and considered in the future.

Based on our results, four groups of meningiomas with distinct prognoses were defined, by a combination of those five features which emerged as independent prognostic factors. Thus, around 20% of the cases were classified as having a very good prognosis in the absence of relapses at 10 years; in contrast, intermediate-2 and high-risk cases displayed a high frequency of relapses with RFS rates at 10-years of 59%±8% and 0%±0%, respectively. To the best of our knowledge, this is one of the largest series of meningiomas reported so far, in which tumor cytogenetics was systematically investigated, together with other relevant clinical and histopathological features of the disease. In line with previous observations by our and other groups [5, 8, 41, 44, 494, 499, 500], WHO grade plus tumor cytogenetics, patient age, tumor size and localization emerged as the most relevant prognostic factors. Of note, once WHO grade I meningiomas were separately considered, the other four parameters retained their independent prognostic value; consequently, the newly proposed scoring system based on these four prognostic factors was also retained, at the expenses of a decreased frequency of patients included in the intermediate-2 and poor-prognosis risk-categories. Altogether,

these results indicate that the new classification here proposed adds valuable prognostic information to that of the WHO grade alone, allowing for a more refined risk-stratification of meningiomas.

Apart from the WHO grade, tumor cytogenetics emerged as being particularly informative. For decades now, the cytogenetic profile of tumor cells has been recurrently associated with the clinical outcome of meningioma patients [61, 62, 78, 223, 495, 497, 498, 501]. In fact, many different individual chromosomal alterations have been associated with the outcome of meningioma patients, this including particularly the losses of chromosomes 1p, 10 and 14 [32, 61, 64, 135]. Of note, monosomy 14 [45, 60, 63, 64, 497, 502] has even emerged as an independent prognostic factor for RFS [45, 60], especially when associated with other specific chromosomal alterations - e.g. del(1p36) [60] -. These latter findings may contribute to explain why in our series the presence of monosomy 14 did not retained its independent prognostic value, as all cases showing monosomy 14 also displayed complex karyotypes, which frequently included del(1p36) (data not shown). Although a higher frequency of complex karyotypes was found among grade II/III *vs.* grade I meningiomas, still a significant fraction of the latter cases showed coexistence of multiple chromosomal alterations in association with a worse outcome, which contributes to explain the independent prognostic value of tumor cytogenetics on top of the WHO grade. Regarding implementation of the cytogenetic studies in routine clinical practice it should be noted that this may be easily achieved by using either unexpensive copy number oligonucleotide arrays (FullChromaArray^{TM}, patent number 201231829) containing probes for the analysis of copy number alteration of the 24 human chromosomes or FISH technique based on a relatively limited number of probes for a few chromosomes (e.g. chromosomes 1p, 7, 14, 18 and 22).

Of note, such prognostic impact of tumor cytogenetics was further confirmed in another independent series of patients in which SNP-arrays were used to assess the CN alteration profile of meningiomas, therefore confirming also our iFISH results.

Other adverse prognostic factors that retained their independent prognostic value in our series included younger age (<55 years), tumor size >50mm and tumor localization at intraventricular and anterior cranial base areas; all such features of meningiomas have been previously described by our and other groups as relevant prognostic factors [5, 8, 41, 44, 494]. However, it should be noted that, while several studies have shown a worse outcome for younger patients [41, 59, 503], others were not able to confirm the prognostic value of patient age [47, 504]. In turn, it should also be highlighted that the mechanisms underlying the adverse impact on RFS of a higher tumor size and both an intraventricular and an anterior cranial base localization, still remain to be elucidated. Despite this, it could be hypothesized

that a greater tumor size as well as specific tumor localizations, could be associated with patterns of local tissue tumor infiltration (and behavior) that may lead to a lower probability of achieving complete tumor resection. In this regard, tumors localized in the convexity are typically considered to be curable by surgical resection [59, 505, 506], while intraventricular and skull-based meningiomas (especially those localized in the petroclival region and those that show involvement of the cavernous sinus and the orbit) are associated with a more unfavorable outcome, due to the need to use more complex resection procedures to prevent neurological sequelae [5, 8]. Despite this, it should be noted that in other series, parasagittal/falcine [507, 508] and non-skull base tumors [44, 496, 500] have also been associated with a worse prognosis. Therefore, the specific reasons for the association here described between tumor localization and patient prognosis deserves further investigations. In this regard, it should be noted that in our series, only a small percentage of cases did not undergo complete tumor resection, no significant correlation being found for these cases between tumor localization and recurrence; in fact, this small group of cases showed an apparently similar distribution by localization, to that observed for the other tumors.

Most interestingly, our results also indicate that the new prognostic scoring system here proposed clearly improves the predictive value of tumor grade, as well as of other previously proposed prognostic scoring systems (e.g. the Maillo et al. score [45]), particularly as regards the identification of a subgroup of meningiomas with a very good-prognosis who remain relapse-free at 10 years and that would potentially not require close monitoring; in addition, it also allows the identification of a small group of patients which mainly included grade II/III tumors together with a few grade I meningiomas, who show a dismal outcome with a recurrence rate at 10 years of 100%±0%, for whom closer monitoring and/or alternative treatment strategies are required.

In summary, here we propose a new prognostic classification for meningioma patients based on tumor size, localization and cytogenetics, in addition to patient age and the WHO grade. Such classification allows stratification of meningioma patients at diagnosis into four risk categories associated with significantly different relapse rates, and that may potentially benefit from different follow-up strategies, as well as distinct treatment approaches.

Chapter VIII – Concluding Remarks

Tumor development is a complex multistep process. Such process usually requires sequential accumulation of specific genetic and/or epigenetic alterations in the neoplastic cell, as well as the development of a microenvironment that would favor the settlement and growth of the tumor. This implies the establishment of a complex network of bidirectional communications between the tumor cells and the surrounding non-tumorigenic cells infiltrating the tumor.

During the past few decades, many studies have investigated the genetics of meningiomas and significant advances have been made in the identification of the most common cytogenetic alterations, and their associated targeted genes [3, 4, 8]. In several of these reports, an accumulation of chromosomal alterations within the more advanced WHO histopathological grades (grade II and III tumors) has been shown. In addition, these studies also showed that monosomy 22/*NF2* mutations could potentially represent one of the most common first chromosomal alterations to occur in meningiomas [3, 129]. In parallel, WHO grade I/benign meningiomas have been shown to present with multiple pathways of intratumoral cytogenetic evolution including those initiated by monosomy 22/*NF2* mutations [224]. Therefore, as a whole, meningiomas comprise a genetically heterogeneous group of tumors, such genetic diversity being observed already at the early stages, e.g. among histologically benign/grade I meningiomas, in association with an equally wide range of biological features and clinical outcomes [73]. Overall, three major cytogenetic profiles of meningiomas have been identified so far, namely (i) diploid meningiomas, (ii) tumors showing isolated monosomy 22/del(22q) and (iii) meningiomas with complex karyotypes. From the prognostic point of view, meningiomas with a diploid karyotype and cases with isolated monosomy 22/del(22q) have a particularly good prognosis, while tumors with more complex karyotypes, specially those carrying del(1p36) and/or monosomy 14, display a significantly worse outcome due to a significantly higher risk of recurrence [73].

In contrast to all the information available about cytogenetic alterations of meningiomas, current knowledge about the tumor-infiltrating cells, including the immune cells present in the meningioma microenvironment, as well as their role in the development of the tumor, remains very limited. Despite this, it should be noted that the different effects that the surrounding (e.g. immune) cells might have in the behavior of the tumor, e.g. through their support of tumor growth or control of the disease, is already well established [446, 448]; in addition, tumor infiltration by immune cells (e.g. macrophages) has been recurrently reported in meningiomas [272, 274, 276, 348]. In this doctoral thesis we evaluated the genetic alterations of meningiomas as well as the distribution, phenotypic and functional features of the immune cells present in the microenvironment of these tumors.

First, we analyzed the overall cellular composition and immunophenotypic profile of the major cell populations coexisting in meningioma (tumor) samples, using MFC immunophenotyping. For this purpose, a broad panel of markers was applied to the analysis of single cell suspensions prepared from the tumor samples, devoted to the identification and characterization of both neoplastic and infiltrating immune cells. Overall, our results confirmed the heterogeneous and variable cellular composition of meningiomas. Thus, together with a major fraction of neoplastic cells, meningiomas systematically showed variable levels of infiltration by tissue macrophages and several different lymphocyte subsets. Identification of the two major groups of cells - neoplastic and infiltrating immune cells – in the tumor, was achieved through their distinct granularity and their $CD45^-$ *vs.* $CD45^+$ immunophenotypic profile. In addition, differential MFC expression of CD45, HLA-DR and CD44 emerged as the optimal combination of markers for further identification of all other major cell populations (e.g. tumor cells, tissue macrophages and lymphocytes) within meningioma tissues, a more detailed characterization of these distinct cell populations requiring additional markers. To the best of our knowledge, this is the first study to provide a detailed immunophenotypic profile of individual meningioma cells and cell populations.

Among $CD45^+$ cells, the majority of cells showed an HLA-$DR^{hi}CD14^+CD68^+CD44^{-/+}CD16^{-/+}CD33^{-/+}$ immunophenotype, suggesting a monocytic/macrophage lineage origin, as confirmed by their cytomorphological characteristics and their high phagocytic and endocytic capabilities. In addition, our results provided additional insight into the phenotypic and functional properties of such macrophages (TiMa); thereby, TiMa showed expression of proteins involved in immune regulation mechanisms (e.g. HLA-I major histocompatibility complex and the CD55 and CD59 complement regulatory proteins), the CD13/APN and CD38 ectoenzymes, the anti-apoptotic bcl-2 protein, activation induced markers such as CD69, and multiple adhesion molecules (CD2, CD44, CD58 and CD99) and tetraspanins (CD9, CD53, CD63, CD81 and CD37). The other $CD45^+$ cells infiltrating the tumor corresponded to $CD45^{hi}$ lymphocytes which were systematically found at lower numbers; the majority of tumor-infiltrating lymphocytes corresponded to cytotoxic T $CD8^+$ and NK cells, with just a few B cells. Treg infiltration, evaluated by the presence of $CD4^+CD25^{hi}CD127^{lo}$ cells, was found at very low levels in the majority of meningioma samples. This, together with the presence of the CD28 co-stimulatory molecule on most T cells, and the greater expression of the CD69 activation-associated marker, point out to local occurrence of a T/NK cell activation in the absence of immune tolerance induced by Tregs. Consequently, the overall immunophenotype of the immune infiltrates in meningiomas suggest a potential role of immune infiltrating cells in controlling tumor growth,

in contrast to what has been reported in other more malignant brain tumors, such as gliomas [327, 370].

$CD45^-$ meningioma cells showed expression of several adhesion-associated molecules which may play an important role in the regulation of tumor cell motility, proliferation and intracellular signaling (e.g. APN/CD13 ectoenzyme, CD44, CD9, CD63 and CD81 tetraspanins [92, 375, 415, 416, 419-422]); in addition, they also displayed high levels of expression of the CD55 and CD59 complement regulatory molecules, and they had high levels of the HLA-I major histocompatibility complex molecule on their surface. Overall, these results suggest that tumor cells from meningiomas are highly-protected from complement-mediated lysis [429, 430], at the same time they could facilitate immunesurveillance and control of tumor growth through increased HLA-I expression, a molecule involved in presentation of self tumor antigens to cytotoxic T and NK cells [431]. Meningioma tumor cells also showed heterogeneous patterns of expression of growth factor receptors (e.g. HER2/neu, IGFR, PDGFRβ and EGFR) and the anti-apoptotic bcl-2 protein, a group of molecules involved in signaling pathways that control meningioma cell growth and survival [156, 310, 423-428].

Most interestingly, both the distribution of the different types of immune cells in the tumor samples analyzed, as well as the immunophenotypic profile of meningioma tumor cells, proved to be associated with other relevant features of the disease, particularly the cytogenetic and gene expression profiles of tumor cells. Accordingly, diploid meningiomas were typically characterized by high expression of the CD55 (DAF) complement regulatory protein on the membrane of neoplastic cells. Since high cellular expression of complement regulatory proteins is associated with resistance to complement-mediated cytotoxicity and usually with more aggressive tumor features [474], our findings would support an important role for this protein in this cytogenetic subgroup of meningiomas. In addition, high protein and mRNA expression levels of aminopeptidase N (CD13) were also found in diploid meningiomas, suggesting that this cell surface protease could also play a unique role in this cytogenetic group of meningiomas [414, 419]. Of note, both overexpressed proteins may lead to a decreased interaction of tumor cells with the immunological microenvironment. In line with this, diploid tumors also emerged as the cytogenetic subgroup of meningiomas with lowest levels of infiltration by TiMa and lymphocytes, suggesting that lack of major cytogenetic alterations in meningiomas is also associated with no major alterations in the tumor immunogenicity and/or microenvironment.

In contrast to diploid meningiomas, most meningiomas carrying isolated monosomy 22/del(22q) showed very high levels of infiltration by TiMa, and to a less extent also, NK cells. In turn, this was associated with a unique protein (and mRNA) expression profile, with

particularly high levels of PDGFRβ and bcl2. These findings point out the potential relevance of extracellular signaling through e.g. PDGFRβ [175], and inhibition of apoptosis [310, 423, 476] (e.g. through activation of Akt [477, 478]), in supporting the growth of tumors with isolated monosomy 22/del(22q). Extracellular signaling could be specifically associated with a unique microenvironment since amplification of *PDGFRB* at the genetic level was not found in these tumors. In line with this hypothesis, TiMa from meningiomas carrying isolated monosomy 22/del(22q) also displayed a more activated and functionally matured phenotype, as reflected by higher expression levels of the CD69 and CD63 activation markers and the maturation-associated FcγRIII receptor (CD16) [451, 452]; increased expression of the CD44 and CD9 adhesion molecules, involved in the recruitment and activation of macrophages [397, 454, 456], was also observed on TiMa from these *vs.* other meningiomas. Altogether, these findings suggest that TiMa may play a central role in controlling tumor growth as well as in promoting homing/chemoattraction of other types of immune cells into the tumor, in meningiomas with isolated monosomy 22/del(22q). The greater numbers of infiltrating TiMa and cytotoxic NK cells, together with the greater expression of the CD69 early-activation antigen on the infiltrating lymphocytes, further support an activation of immune surveillance mechanisms in this cytogenetic subgroup of meningiomas, for the elimination of tumor cells via cytotoxic mechanisms. In line with the MFC data, GEP of meningiomas with isolated monosomy 22/del(22q) confirmed an increased inflammatory and immune response consisting of greater expression of genes involved in antigen presentation (e.g. HLA and HLA-associated molecules), phagocytosis (CD16, CD32, CD64 Fc receptors) and cell activation/cell signaling (e.g. immune co-stimulatory molecules – CD86 -, toll-like receptors and inflammatory cytokines) in meningiomas with isolated monosomy 22/del(22q). In addition, GEP data also support a polarization towards an M1 *vs.* M2-macrophage phenotype, and consequently also, a more favorable anti-tumoral microenvironment. The greater numbers of NK cells found in this subgroup of meningiomas would further support this hypothesis [286, 459]. Altogether, these results may help to explain the benign outcome of the greater majority of patients with meningioma carrying isolated monosomy 22/del(22q) compared to e.g. complex karyotype cases. Although the specific mechanisms underlying the above reported associations remain to be elucidated, it might be hypothesized that loss of expression of genes specifically coded in chromosome 22 might be directly related with the unique pattern of immune infiltration of meningiomas with isolated monosomy 22/del(22q). Loss of the *MIF* gene coded at 22q11.23 could play an important role in this subgroup of meningiomas. MIF was initially described as a macrophage migration inhibitory factor and it is currently known to have more broad e.g. inflammatory, anti-apoptotic and proliferative functions and to promote tumor growth and

progression [463, 464]. In line with this, Huang et al. [509] have recently reported that increased co-expression of MIF and MMP9 is associated with tumor recurrence of meningioma. However, further investigations are necessary to confirm the pathogenic role of MIF in the association here reported between monosomy 22/del(22q) cases and a greater tumor infiltration by macrophages.

Finally, cytogenetically complex meningiomas emerged as those meningiomas showing the highest proliferation index in association with unique levels of expression of several proteins involved in the control of tumor cell proliferation and invasion and the most heterogeneous patterns of infiltration by immune cells. Thus, complex meningiomas showed low expression levels of aminopeptidase N (CD13), in line with what has been previously reported by Mawrin et al. [419] who showed reduced mRNA and protein expression levels of this protein as well as of its enzymatic activity, in high-grade meningiomas; in addition, meningiomas displaying complex karyotypes also had decreased expression of the CD9 and CD81 tetraspanins, a phenotype that has been associated with malignant progression [480], and low expression of the HER2/neu growth factor receptor on the cell membrane, potentially reflecting a higher cell activation and protein processing on the tumor cell surface [483-485]. Regarding immune infiltrates, it should be noted that despite a fraction of all tumors carrying complex karyotypes displayed high level of infiltration by TiMa, in these cases, macrophages displayed higher levels of expression of CD206 *vs.* those of meningiomas having isolated monosomy 22/del(22q); in addition, such cases also showed higher numbers of $CD4^{+}CD25^{hi}CD127^{-/lo}$ Treg cells. These results suggest a switch in the functional phenotype of macrophages towards an M2-profile among cases with complex karyotypes and high TiMa infiltration, which would contribute to support tumor growth/progression and recurrence, at the same time it would also lead to a poorer prognosis.

In summary, regarding association between tumor cytogenetics and the tumoral microenvironment, it might be concluded that while diploid tumors show a rather preserved phenotype of tumor cells with low levels of infiltration by immune cells, presence of isolated monosomy 22/del(22q) would lead to a more inflammatory phenotype of the tumor, potentially associated with a more effective anti-tumor immune surveillance. Acquisition of a complex karyotype would in turn lead to a more aggressive phenotype, associated or not with a pro-tumoral inflammatory response in the tumor microenvironment. Despite this and the adverse prognostic impact of higher expression of both CD44 and CD53 on the patients' relapse-free survival, none of these parameters had an independent prognostic value, which could be due to their close association with tumor cytogenetics. Further investigations in larger

series of patients, in which extended antibody panels are used, are required to confirm this hypothesis.

Tumor recurrence still remains the major clinical complication of meningioma, even among benign/WHO grade I tumors. In the past, multiple prognostic factors and some prognostic classifications have been proposed for meningiomas, cytogenetics emerging as one of the most relevant predictors for tumor recurrence, among other factors, e.g. patient sex and age and tumor size and localization [8, 32, 41, 44, 62, 223, 495, 499]. However, at present, assessment of the risk of recurrence of meningiomas still remains a challenge, particularly in grade I tumors, among which around half of all recurrences occur. Despite the major advances in the understanding of the genetic alterations of meningiomas, this information has still not translated into the classification and management of the patients in routine clinical practice. Overall, this points out to the need for more refined prognostic criteria, to improve the predictive value of the current histopathological classification, particularly among benign/grade I meningiomas.

Therefore, another major goal of this work was to construct a prognostic scoring system for risk stratification of meningioma patients, based on the most relevant clinical and biological features of the disease, including tumor cytogenetics. For this purpose, a large series of meningioma patients followed for rather long periods of time and in whom information about all potentially relevant prognostic parameters was systematically assessed at diagnosis, was analyzed. Overall, five distinct parameters emerged as independent prognostic factors, i.e. patient age, tumor localization and size in addition to the WHO grade and tumor cytogenetics. In more detail, complex karyotypes defined by the presence of ≥2 chromosomal alterations emerged as an independent adverse prognostic factor, together with younger age (<55 years), tumor size >50mm and tumor localization at the intraventricular and anterior cranial base areas. Once these prognostic factors were combined into a prognostic scoring system, meningioma patients could be stratified into four different risk categories with significantly distinct recurrence-free survival rates. These included a significant group of very low-risk cases who would not require follow-up, together with a small group of poor-prognosis cases where additional/alternative therapeutic measures are required and should be prospectively considered in the near future. Most importantly, when WHO grade I meningiomas were separately considered, the remaining four prognostic variables (all five described above except the WHO grade) retained their independent prognostic value, their combination into the proposed scoring system also allowing discrimination of WHO grade I cases into distinct risk categories with significantly different recurrence rates. Further prospective studies are

welcome now to validate this new prognostic classification of meningiomas in independent patient series.

In summary, in the present work we confirmed that meningiomas are relatively heterogeneous tumors, not only from the clinical but also the genetic and biological point of view. Such biological heterogeneity of meningiomas was also confirmed for the first time at the tumor microenvironmental level. In this regard, analysis of the cellular composition of meningioma samples showed systematic presence of variable numbers of infiltrating immune cells coexisting with the neoplastic cells, such immune infiltrates mainly consisting of tissue macrophages and to a lesser extent also, cytotoxic $TCD8^+$ and NK cells. Furthermore, we showed that both the protein expression profile of neoplastic cells and the levels of infiltration by the immune cells, as evaluated by MFC immunophenotyping, are closely associated with tumor cytogenetics, which may reflect the involvement of different intra and extracellular signaling pathways in the distinct cytogenetic subgroups of meningiomas. Interestingly, cases carrying isolated monosomy 22/del(22q) were specifically associated with increased infiltration of the tumor by tissue macrophages, NK cells and activated lymphocytes, which may contribute to a better control of the disease, ultimately leading to a better outcome of this patient group. Whether the different levels of immune infiltrates are directly related to the altered expression of specific genes due to concurrent cytogenetic alterations deserves further investigations. Despite this, it should be noted that the prognostic significance of the levels of infiltration of the tumor by the distinct subpopulations of immune cells was limited and not independent from tumor cytogenetics. In this regard, in the last part of our study we built and proposed a new prognostic classification for meningioma patients based on tumor grade and cytogenetics plus clinical parameters with an independent predictive value for tumor recurrence-free survival, namely patient age, tumor size and localization. The prognostic classification proposed here allows stratification of meningioma patients already at diagnosis, into distinct risk categories associated with significantly different relapse rates, and who may potentially benefit from different follow-up strategies and/or distinct treatment approaches.

References

1. Ostrom, Q.T., et al., *CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010.* Neuro Oncol, 2013. **15 Suppl 2**: p. ii1-56.
2. Rigau, V., et al., *French brain tumor database: 5-year histological results on 25 756 cases.* Brain Pathol, 2011. **21**(6): p. 633-44.
3. Riemenschneider, M.J., A. Perry, and G. Reifenberger, *Histological classification and molecular genetics of meningiomas.* Lancet Neurol, 2006. **5**(12): p. 1045-54.
4. Mawrin, C. and A. Perry, *Pathological classification and molecular genetics of meningiomas.* J Neurooncol, 2010. **99**(3): p. 379-91.
5. Marosi, C., et al., *Meningioma.* Crit Rev Oncol Hematol, 2008. **67**(2): p. 153-71.
6. Perry, A., et al., *Meningiomas*, in *WHO Classification of Tumors of the Central Nervous System.*, D.N. Louis, et al., Editors. 2007, IARC press: Lyon, France. p. 164-72.
7. Larjavaara, S., et al., *Is the incidence of meningiomas underestimated? A regional survey.* Br J Cancer, 2008. **99**(1): p. 182-4.
8. Saraf, S., B.J. McCarthy, and J.L. Villano, *Update on meningiomas.* Oncologist, 2011. **16**(11): p. 1604-13.
9. Wiemels, J., M. Wrensch, and E.B. Claus, *Epidemiology and etiology of meningioma.* J Neurooncol, 2010. **99**(3): p. 307-14.
10. Neglia, J.P., et al., *New primary neoplasms of the central nervous system in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study.* J Natl Cancer Inst, 2006. **98**(21): p. 1528-37.
11. Hijiya, N., et al., *Cumulative incidence of secondary neoplasms as a first event after childhood acute lymphoblastic leukemia.* JAMA, 2007. **297**(11): p. 1207-15.
12. Claus, E.B., et al., *Dental x-rays and risk of meningioma.* Cancer, 2012. **118**(18): p. 4530-7.
13. Sadetzki, S., et al., *Radiation-induced meningioma: a descriptive study of 253 cases.* J Neurosurg, 2002. **97**(5): p. 1078-82.
14. Preston, D.L., et al., *Tumors of the nervous system and pituitary gland associated with atomic bomb radiation exposure.* J Natl Cancer Inst, 2002. **94**(20): p. 1555-63.
15. Phillips, L.E., et al., *History of head trauma and risk of intracranial meningioma: population-based case-control study.* Neurology, 2002. **58**(12): p. 1849-52.
16. Eskandary, H., et al., *Incidental findings in brain computed tomography scans of 3000 head trauma patients.* Surg Neurol, 2005. **63**(6): p. 550-3; discussion 553.
17. Chargari, C., et al., *Reapprasial of the role of endocrine therapy in meningioma management.* Endocr Relat Cancer, 2008. **15**(4): p. 931-41.
18. Vranic, A., M. Peyre, and M. Kalamarides, *New insights into meningioma: from genetics to trials.* Curr Opin Oncol, 2012. **24**(6): p. 660-5.
19. Custer, B.S., T.D. Koepsell, and B.A. Mueller, *The association between breast carcinoma and meningioma in women.* Cancer, 2002. **94**(6): p. 1626-35.
20. Lee, E., et al., *Association of meningioma with reproductive factors.* Int J Cancer, 2006. **119**(5): p. 1152-7.
21. Wigertz, A., et al., *Risk of brain tumors associated with exposure to exogenous female sex hormones.* Am J Epidemiol, 2006. **164**(7): p. 629-36.
22. Claus, E.B., et al., *Exogenous hormone use and meningioma risk: what do we tell our patients?* Cancer, 2007. **110**(3): p. 471-6.
23. Claus, E.B., et al., *Exogenous hormone use, reproductive factors, and risk of intracranial meningioma in females.* J Neurosurg, 2013. **118**(3): p. 649-56.
24. Fathi, A.R. and U. Roelcke, *Meningioma.* Curr Neurol Neurosci Rep, 2013. **13**(4): p. 337.
25. Saloner, D., et al., *Modern meningioma imaging techniques.* J Neurooncol, 2010. **99**(3): p. 333-40.
26. Rockhill, J., M. Mrugala, and M.C. Chamberlain, *Intracranial meningiomas: an overview of diagnosis and treatment.* Neurosurg Focus, 2007. **23**(4): p. E1.

27. Hasseleid, B.F., et al., *Surgery for convexity meningioma: Simpson Grade I resection as the goal: clinical article.* J Neurosurg, 2012. **117**(6): p. 999-1006.
28. Mirimanoff, R.O., et al., *Meningioma: analysis of recurrence and progression following neurosurgical resection.* J Neurosurg, 1985. **62**(1): p. 18-24.
29. Goldsmith, B.J., et al., *Postoperative irradiation for subtotally resected meningiomas. A retrospective analysis of 140 patients treated from 1967 to 1990.* J Neurosurg, 1994. **80**(2): p. 195-201.
30. Santacroce, A., et al., *Long-term tumor control of benign intracranial meningiomas after radiosurgery in a series of 4565 patients.* Neurosurgery, 2012. **70**(1): p. 32-9; discussion 39.
31. Chamberlain, M.C., *Hydroxyurea for recurrent surgery and radiation refractory high-grade meningioma.* J Neurooncol, 2012. **107**(2): p. 315-21.
32. Yew, A., et al., *Chromosomal alterations, prognostic factors, and targeted molecular therapies for malignant meningiomas.* J Clin Neurosci, 2013. **20**(1): p. 17-22.
33. Wen, P.Y., et al., *Phase II study of imatinib mesylate for recurrent meningiomas (North American Brain Tumor Consortium study 01-08).* Neuro Oncol, 2009. **11**(6): p. 853-60.
34. Reardon, D.A., et al., *Phase II study of Gleevec(R) plus hydroxyurea (HU) in adults with progressive or recurrent meningioma.* J Neurooncol, 2012. **106**(2): p. 409-15.
35. Lou, E., et al., *Bevacizumab therapy for adults with recurrent/progressive meningioma: a retrospective series.* J Neurooncol, 2012. **109**(1): p. 63-70.
36. Goutagny, S., et al., *Radiographic regression of cranial meningioma in a NF2 patient treated by bevacizumab.* Ann Oncol, 2011. **22**(4): p. 990-1.
37. Grunberg, S.M., et al., *Long-term administration of mifepristone (RU486): clinical tolerance during extended treatment of meningioma.* Cancer Invest, 2006. **24**(8): p. 727-33.
38. Chamberlain, M.C. and M.J. Glantz, *Interferon-alpha for recurrent World Health Organization grade 1 intracranial meningiomas.* Cancer, 2008. **113**(8): p. 2146-51.
39. Muhr, C., et al., *Meningioma treated with interferon-alpha, evaluated with [(11)C]-L-methionine positron emission tomography.* Clin Cancer Res, 2001. **7**(8): p. 2269-76.
40. Chamberlain, M.C., M.J. Glantz, and C.E. Fadul, *Recurrent meningioma: salvage therapy with long-acting somatostatin analogue.* Neurology, 2007. **69**(10): p. 969-73.
41. Stafford, S.L., et al., *Primarily resected meningiomas: outcome and prognostic factors in 581 Mayo Clinic patients, 1978 through 1988.* Mayo Clin Proc, 1998. **73**(10): p. 936-42.
42. Yang, S.Y., et al., *Atypical and anaplastic meningiomas: prognostic implications of clinicopathological features.* J Neurol Neurosurg Psychiatry, 2008. **79**(5): p. 574-80.
43. McGovern, S.L., et al., *A comparison of World Health Organization tumor grades at recurrence in patients with non-skull base and skull base meningiomas.* J Neurosurg, 2010. **112**(5): p. 925-33.
44. Zhou, P., et al., *Three risk factors for WHO grade II and III meningiomas: A study of 1737 cases from a single center.* Neurol India, 2013. **61**(1): p. 40-4.
45. Maillo, A., et al., *New classification scheme for the prognostic stratification of meningioma on the basis of chromosome 14 abnormalities, patient age, and tumor histopathology.* J Clin Oncol, 2003. **21**(17): p. 3285-95.
46. Oya, S., et al., *Significance of Simpson grading system in modern meningioma surgery: integration of the grade with MIB-1 labeling index as a key to predict the recurrence of WHO Grade I meningiomas.* J Neurosurg, 2012. **117**(1): p. 121-8.
47. Kasuya, H., et al., *Clinical and radiological features related to the growth potential of meningioma.* Neurosurg Rev, 2006. **29**(4): p. 293-6.
48. Maillo, A., et al., *Proportion of S-phase tumor cells measured by flow cytometry is an independent prognostic factor in meningioma tumors.* Cytometry, 1999. **38**(3): p. 118-23.

49. Simon, M., et al., *Telomerase activity and expression of the telomerase catalytic subunit, hTERT, in meningioma progression.* J Neurosurg, 2000. **92**(5): p. 832-40.
50. Chen, H.J., et al., *Implication of telomerase activity and alternations of telomere length in the histologic characteristics of intracranial meningiomas.* Cancer, 2000. **89**(10): p. 2092-8.
51. Langford, L.A., et al., *Telomerase activity in ordinary meningiomas predicts poor outcome.* Hum Pathol, 1997. **28**(4): p. 416-20.
52. Maes, L., et al., *The hTERT-protein and Ki-67 labelling index in recurrent and non-recurrent meningiomas.* Cell Prolif, 2005. **38**(1): p. 3-12.
53. Maes, L., et al., *Telomerase activity and hTERT protein expression in meningiomas: an analysis in vivo vs. in vitro.* Anticancer Res, 2006. **26**(3B): p. 2295-300.
54. Tseng, K.Y., et al., *Osteopontin expression is a valuable marker for prediction of short-term recurrence in WHO grade I benign meningiomas.* J Neurooncol, 2010. **100**(2): p. 217-23.
55. Preusser, M., et al., *Microvascularization and expression of VEGF and its receptors in recurring meningiomas: pathobiological data in favor of anti-angiogenic therapy approaches.* Clin Neuropathol, 2012. **31**(5): p. 352-60.
56. Markovic, M., et al., *Prognostic value of peritumoral edema and angiogenesis in intracranial meningioma surgery.* J BUON, 2013. **18**(2): p. 430-6.
57. Lee, S.H., et al., *Significance of COX-2 and VEGF expression in histopathologic grading and invasiveness of meningiomas.* APMIS, 2014. **122**(1): p. 16-24.
58. Barresi, V. and G. Tuccari, *Increased ratio of vascular endothelial growth factor to semaphorin3A is a negative prognostic factor in human meningiomas.* Neuropathology, 2010.
59. Ruiz, J., et al., *Clinicopathological variables, immunophenotype, chromosome 1p36 loss and tumour recurrence of 247 meningiomas grade I and II.* Histol Histopathol, 2010. **25**(3): p. 341-9.
60. Maillo, A., et al., *Early recurrences in histologically benign/grade I meningiomas are associated with large tumors and coexistence of monosomy 14 and del(1p36) in the ancestral tumor cell clone.* Neuro Oncol, 2007. **9**(4): p. 438-46.
61. Kim, Y.J., et al., *Histopathologic indicators of recurrence in meningiomas: correlation with clinical and genetic parameters.* Virchows Arch, 2006. **449**(5): p. 529-38.
62. Ketter, R., et al., *Predictive value of progression-associated chromosomal aberrations for the prognosis of meningiomas: a retrospective study of 198 cases.* J Neurosurg, 2001. **95**(4): p. 601-7.
63. Tabernero, M.D., et al., *Characterization of chromosome 14 abnormalities by interphase in situ hybridization and comparative genomic hybridization in 124 meningiomas: correlation with clinical, histopathologic, and prognostic features.* Am J Clin Pathol, 2005. **123**(5): p. 744-51.
64. Cai, D.X., et al., *Chromosome 1p and 14q FISH analysis in clinicopathologic subsets of meningioma: diagnostic and prognostic implications.* J Neuropathol Exp Neurol, 2001. **60**(6): p. 628-36.
65. Mihaila, D., et al., *Meningiomas: loss of heterozygosity on chromosome 10 and marker-specific correlations with grade, recurrence, and survival.* Clin Cancer Res, 2003. **9**(12): p. 4443-51.
66. Leuraud, P., et al., *Prognostic value of allelic losses and telomerase activity in meningiomas.* J Neurosurg, 2004. **100**(2): p. 303-9.
67. Perry, A., et al., *A role for chromosome 9p21 deletions in the malignant progression of meningiomas and the prognosis of anaplastic meningiomas.* Brain Pathol, 2002. **12**(2): p. 183-90.

68. Maillo, A., et al., *Gains of chromosome 22 by fluorescence in situ hybridization in the context of an hyperdiploid karyotype are associated with aggressive clinical features in meningioma patients.* Cancer, 2001. **92**(2): p. 377-85.
69. Jansen, M., et al., *Gain of chromosome arm 1q in atypical meningioma correlates with shorter progression-free survival.* Neuropathol Appl Neurobiol, 2012. **38**(2): p. 213-9.
70. Gabeau-Lacet, D., et al., *Genomic profiling of atypical meningiomas associates gain of 1q with poor clinical outcome.* J Neuropathol Exp Neurol, 2009. **68**(10): p. 1155-65.
71. Perez-Magan, E., et al., *Differential expression profiling analyses identifies downregulation of 1p, 6q, and 14q genes and overexpression of 6p histone cluster 1 genes as markers of recurrence in meningiomas.* Neuro Oncol, 2010. **12**(12): p. 1278-90.
72. Perez-Magan, E., et al., *Genetic alterations associated with progression and recurrence in meningiomas.* J Neuropathol Exp Neurol, 2012. **71**(10): p. 882-93.
73. Tabernero, M.D., et al., *Gene expression profiles of meningiomas are associated with tumor cytogenetics and patient outcome.* Brain Pathol, 2009. **19**(3): p. 409-20.
74. Serna, E., et al., *Gene expression profiles of metabolic aggressiveness and tumor recurrence in benign meningioma.* PLoS One, 2013. **8**(6): p. e67291.
75. Lee, Y., et al., *Genomic landscape of meningiomas.* Brain Pathol, 2010. **20**(4): p. 751-62.
76. Pham, M.H., et al., *Molecular genetics of meningiomas: a systematic review of the current literature and potential basis for future treatment paradigms.* Neurosurg Focus, 2011. **30**(5): p. E7.
77. Choy, W., et al., *The molecular genetics and tumor pathogenesis of meningiomas and the future directions of meningioma treatments.* Neurosurg Focus, 2011. **30**(5): p. E6.
78. Zang, K.D., *Meningioma: a cytogenetic model of a complex benign human tumor, including data on 394 karyotyped cases.* Cytogenet Cell Genet, 2001. **93**(3-4): p. 207-20.
79. Espinosa, A.B., et al., *The cytogenetic relationship between primary and recurrent meningiomas points to the need for new treatment strategies in cases at high risk of relapse.* Clin Cancer Res, 2006. **12**(3 Pt 1): p. 772-80.
80. Al-Mefty, O., et al., *Malignant progression in meningioma: documentation of a series and analysis of cytogenetic findings.* J Neurosurg, 2004. **101**(2): p. 210-8.
81. Zang, K.D. and H. Singer, *Chromosomal consitution of meningiomas.* Nature, 1967. **216**(5110): p. 84-5.
82. Zankl, H. and K.D. Zang, *Cytological and cytogenetical studies on brain tumors. 4. Identification of the missing G chromosome in human meningiomas as no. 22 by fluorescence technique.* Humangenetik, 1972. **14**(2): p. 167-9.
83. Ruttledge, M.H., et al., *Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas.* Nat Genet, 1994. **6**(2): p. 180-4.
84. Alexiou, G.A., et al., *Genetic and molecular alterations in meningiomas.* Clin Neurol Neurosurg, 2011. **113**(4): p. 261-7.
85. Pecina-Slaus, N., *Merlin, the NF2 gene product.* Pathol Oncol Res, 2013. **19**(3): p. 365-73.
86. Knudson, A.G., Jr., *Mutation and cancer: statistical study of retinoblastoma.* Proc Natl Acad Sci U S A, 1971. **68**(4): p. 820-3.
87. Tabernero, M., et al., *Association between mutation of the NF2 gene and monosomy 22 in menopausal women with sporadic meningiomas.* BMC Med Genet, 2013. **14**(1): p. 114.
88. Lomas, J., et al., *Genetic and epigenetic alteration of the NF2 gene in sporadic meningiomas.* Genes Chromosomes Cancer, 2005. **42**(3): p. 314-9.
89. Hansson, C.M., et al., *Comprehensive genetic and epigenetic analysis of sporadic meningioma for macro-mutations on 22q and micro-mutations within the NF2 locus.* BMC Genomics, 2007. **8**: p. 16.
90. van Tilborg, A.A., et al., *Lack of genetic and epigenetic changes in meningiomas without NF2 loss.* J Pathol, 2006. **208**(4): p. 564-73.

91. He, S., et al., *A review of epigenetic and gene expression alterations associated with intracranial meningiomas.* Neurosurg Focus, 2013. **35**(6): p. E5.
92. Stamenkovic, I. and Q. Yu, *Merlin, a "magic" linker between extracellular cues and intracellular signaling pathways that regulate cell motility, proliferation, and survival.* Curr Protein Pept Sci, 2010. **11**(6): p. 471-84.
93. Lallemand, D., et al., *NF2 deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions.* Genes Dev, 2003. **17**(9): p. 1090-100.
94. Lallemand, D., et al., *Merlin regulates transmembrane receptor accumulation and signaling at the plasma membrane in primary mouse Schwann cells and in human schwannomas.* Oncogene, 2009. **28**(6): p. 854-65.
95. McClatchey, A.I., et al., *Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors.* Genes Dev, 1998. **12**(8): p. 1121-33.
96. James, M.F., et al., *The neurofibromatosis 2 protein product merlin selectively binds F-actin but not G-actin, and stabilizes the filaments through a lateral association.* Biochem J, 2001. **356**(Pt 2): p. 377-86.
97. Morrison, H., et al., *The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44.* Genes Dev, 2001. **15**(8): p. 968-80.
98. Manetti, M.E., et al., *Stability of the tumor suppressor merlin depends on its ability to bind paxillin LD3 and associate with beta1 integrin and actin at the plasma membrane.* Biol Open, 2012. **1**(10): p. 949-57.
99. Morrow, K.A. and L.A. Shevde, *Merlin: the wizard requires protein stability to function as a tumor suppressor.* Biochim Biophys Acta, 2012. **1826**(2): p. 400-6.
100. Bai, Y., et al., *Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressor activity of merlin.* Oncogene, 2007. **26**(6): p. 836-50.
101. Li, W., et al., *Merlin: a tumour suppressor with functions at the cell cortex and in the nucleus.* EMBO Rep, 2012. **13**(3): p. 204-15.
102. Hartmann, C., et al., *NF2 mutations in secretory and other rare variants of meningiomas.* Brain Pathol, 2006. **16**(1): p. 15-9.
103. Kros, J., et al., *NF2 status of meningiomas is associated with tumour localization and histology.* J Pathol, 2001. **194**(3): p. 367-72.
104. Evans, J.J., et al., *Molecular alterations in the neurofibromatosis type 2 gene and its protein rarely occurring in meningothelial meningiomas.* J Neurosurg, 2001. **94**(1): p. 111-7.
105. Wellenreuther, R., et al., *Analysis of the neurofibromatosis 2 gene reveals molecular variants of meningioma.* Am J Pathol, 1995. **146**(4): p. 827-32.
106. Clark, V.E., et al., *Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO.* Science, 2013. **339**(6123): p. 1077-80.
107. Peyrard, M., et al., *Characterization of a new member of the human beta-adaptin gene family from chromosome 22q12, a candidate meningioma gene.* Hum Mol Genet, 1994. **3**(8): p. 1393-9.
108. Wozniak, K., et al., *BCR expression is decreased in meningiomas showing loss of heterozygosity of 22q within a new minimal deletion region.* Cancer Genet Cytogenet, 2008. **183**(1): p. 14-20.
109. Barski, D., et al., *Hypermethylation and transcriptional downregulation of the TIMP3 gene is associated with allelic loss on 22q12.3 and malignancy in meningiomas.* Brain Pathol, 2010. **20**(3): p. 623-31.
110. Bello, M.J., et al., *DNA methylation of multiple promoter-associated CpG islands in meningiomas: relationship with the allelic status at 1p and 22q.* Acta Neuropathol, 2004. **108**(5): p. 413-21.
111. Halaka, A.N., et al., *Production of collagenase and inhibitor (TIMP) by intracranial tumors and dura in vitro.* J Neurosurg, 1983. **59**(3): p. 461-6.

112. Das, A., W.L. Tan, and D.R. Smith, *Expression of extracellular matrix markers in benign meningiomas.* Neuropathology, 2003. **23**(4): p. 275-81.
113. Mizoue, T., et al., *Secretion of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by meningiomas detected by cell immunoblot analysis.* Acta Neurochir (Wien), 1999. **141**(5): p. 481-6.
114. Okuducu, A.F., et al., *Ets-1 is up-regulated together with its target gene products matrix metalloproteinase-2 and matrix metalloproteinase-9 in atypical and anaplastic meningiomas.* Histopathology, 2006. **48**(7): p. 836-45.
115. Barresi, V., et al., *MMP-9 expression in meningiomas: a prognostic marker for recurrence risk?* J Neurooncol, 2011. **102**(2): p. 189-96.
116. Okada, M., et al., *Matrix metalloproteinase-2 and matrix metalloproteinase-9 expressions correlate with the recurrence of intracranial meningiomas.* J Neurooncol, 2004. **66**(1-2): p. 29-37.
117. Sayagues, J.M., et al., *Incidence of numerical chromosome aberrations in meningioma tumors as revealed by fluorescence in situ hybridization using 10 chromosome-specific probes.* Cytometry, 2002. **50**(3): p. 153-9.
118. Tabernero, M.D., et al., *Delineation of commonly deleted chromosomal regions in meningiomas by high-density single nucleotide polymorphism genotyping arrays.* Genes Chromosomes Cancer, 2012. **51**(6): p. 606-17.
119. Guan, Y., et al., *Narrowing of the regions of allelic losses of chromosome 1p36 in meningioma tissues by an improved SSCP analysis.* Int J Cancer, 2008. **122**(8): p. 1820-6.
120. Ishino, S., et al., *Loss of material from chromosome arm 1p during malignant progression of meningioma revealed by fluorescent in situ hybridization.* Cancer, 1998. **83**(2): p. 360-6.
121. Bello, M.J., et al., *High-resolution analysis of chromosome arm 1p alterations in meningioma.* Cancer Genet Cytogenet, 2000. **120**(1): p. 30-6.
122. Bostrom, J., et al., *Alterations of the tumor suppressor genes CDKN2A (p16(INK4a)), p14(ARF), CDKN2B (p15(INK4b)), and CDKN2C (p18(INK4c)) in atypical and anaplastic meningiomas.* Am J Pathol, 2001. **159**(2): p. 661-9.
123. Santarius, T., et al., *Molecular analysis of alterations of the p18INK4c gene in human meningiomas.* Neuropathol Appl Neurobiol, 2000. **26**(1): p. 67-75.
124. Piaskowski, S., et al., *GADD45A and EPB41 as tumor suppressor genes in meningioma pathogenesis.* Cancer Genet Cytogenet, 2005. **162**(1): p. 63-7.
125. Nakane, Y., et al., *Malignant transformation-related genes in meningiomas: allelic loss on 1p36 and methylation status of p73 and RASSF1A.* J Neurosurg, 2007. **107**(2): p. 398-404.
126. Lomas, J., et al., *Methylation status of TP73 in meningiomas.* Cancer Genet Cytogenet, 2004. **148**(2): p. 148-51.
127. Muller, P., et al., *Deletion of chromosome 1p and loss of expression of alkaline phosphatase indicate progression of meningiomas.* Clin Cancer Res, 1999. **5**(11): p. 3569-77.
128. Bouvier, C., et al., *Lack of alkaline phosphatase activity predicts meningioma recurrence.* Am J Clin Pathol, 2005. **124**(2): p. 252-8.
129. Weber, R.G., et al., *Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14719-24.
130. Goutagny, S., et al., *Genomic profiling reveals alternative genetic pathways of meningioma malignant progression dependent on the underlying NF2 status.* Clin Cancer Res, 2010. **16**(16): p. 4155-64.
131. Liu, Y., et al., *Aberrant CpG island hypermethylation profile is associated with atypical and anaplastic meningiomas.* Hum Pathol, 2005. **36**(4): p. 416-25.

132. Aydemir, F., et al., *Identification of promoter region methylation patterns of MGMT, CDKN2A, GSTP1, and THBS1 genes in intracranial meningioma patients.* Genet Test Mol Biomarkers, 2012. **16**(5): p. 335-40.
133. Amatya, V.J., Y. Takeshima, and K. Inai, *Methylation of p14(ARF) gene in meningiomas and its correlation to the p53 expression and mutation.* Mod Pathol, 2004. **17**(6): p. 705-10.
134. Simon, M., et al., *Allelic losses on chromosomes 14, 10, and 1 in atypical and malignant meningiomas: a genetic model of meningioma progression.* Cancer Res, 1995. **55**(20): p. 4696-701.
135. Mihaila, D., et al., *Meningiomas: analysis of loss of heterozygosity on chromosome 10 in tumor progression and the delineation of four regions of chromosomal deletion in common with other cancers.* Clin Cancer Res, 2003. **9**(12): p. 4435-42.
136. Joachim, T., et al., *Comparative analysis of the NF2, TP53, PTEN, KRAS, NRAS and HRAS genes in sporadic and radiation-induced human meningiomas.* Int J Cancer, 2001. **94**(2): p. 218-21.
137. Peters, N., et al., *Analysis of the PTEN gene in human meningiomas.* Neuropathol Appl Neurobiol, 1998. **24**(1): p. 3-8.
138. de Robles, P., et al., *Methylation status of MGMT gene promoter in meningiomas.* Cancer Genet Cytogenet, 2008. **187**(1): p. 25-7.
139. Jabini, R., et al., *Pathodiagnostic parameters and evaluation of O- methyl guanine methyl transferase gene promoter methylation in meningiomas.* Gene, 2014.
140. Dobbins, S.E., et al., *Common variation at 10p12.31 near MLLT10 influences meningioma risk.* Nat Genet, 2011. **43**(9): p. 825-7.
141. Lopez-Gines, C., et al., *Association of loss of 1p and alterations of chromosome 14 in meningioma progression.* Cancer Genet Cytogenet, 2004. **148**(2): p. 123-8.
142. Tse, J.Y., et al., *Loss of heterozygosity of chromosome 14q in low- and high-grade meningiomas.* Hum Pathol, 1997. **28**(7): p. 779-85.
143. Zhang, X., et al., *Maternally expressed gene 3, an imprinted noncoding RNA gene, is associated with meningioma pathogenesis and progression.* Cancer Res, 2010. **70**(6): p. 2350-8.
144. Brastianos, P.K., et al., *Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations.* Nat Genet, 2013. **45**(3): p. 285-9.
145. Lusis, E.A., et al., *Integrative genomic analysis identifies NDRG2 as a candidate tumor suppressor gene frequently inactivated in clinically aggressive meningioma.* Cancer Res, 2005. **65**(16): p. 7121-6.
146. Skiriute, D., et al., *Tumor grade-related NDRG2 gene expression in primary and recurrent intracranial meningiomas.* J Neurooncol, 2011. **102**(1): p. 89-94.
147. Buschges, R., et al., *Allelic gain and amplification on the long arm of chromosome 17 in anaplastic meningiomas.* Brain Pathol, 2002. **12**(2): p. 145-53.
148. Arslantas, A., et al., *Comparative genomic hybridization analysis of genomic alterations in benign, atypical and anaplastic meningiomas.* Acta Neurol Belg, 2002. **102**(2): p. 53-62.
149. Surace, E.I., et al., *Functional significance of S6K overexpression in meningioma progression.* Ann Neurol, 2004. **56**(2): p. 295-8.
150. Zhang, M.X., et al., *Constitutive activation of signal transducer and activator of transcription 3 regulates expression of vascular endothelial growth factor in human meningioma differentiation.* J Cancer Res Clin Oncol, 2010. **136**(7): p. 981-8.
151. Johnson, M.D., et al., *Increased STAT-3 and synchronous activation of Raf-1-MEK-1-MAPK, and phosphatidylinositol 3-Kinase-Akt-mTOR pathways in atypical and anaplastic meningiomas.* J Neurooncol, 2009. **92**(2): p. 129-36.
152. Johnson, M.D., et al., *Cerebrospinal fluid stimulates leptomeningeal and meningioma cell proliferation and activation of STAT3.* J Neurooncol, 2012. **107**(1): p. 121-31.

153. Buschges, R., et al., *Analysis of human meningiomas for aberrations of the MADH2, MADH4, APM-1 and DCC tumor suppressor genes on the long arm of chromosome 18.* Int J Cancer, 2001. **92**(4): p. 551-4.
154. Uzum, N. and G.A. Ataoglu, *Histopathological parameters with Ki-67 and bcl-2 in the prognosis of meningiomas according to WHO 2000 classification.* Tumori, 2008. **94**(3): p. 389-97.
155. Verheijen, F.M., et al., *Progesterone receptor, bc1-2 and bax expression in meningiomas.* J Neurooncol, 2002. **56**(1): p. 35-41.
156. Abramovich, C.M. and R.A. Prayson, *Apoptotic activity and bcl-2 immunoreactivity in meningiomas. Association with grade and outcome.* Am J Clin Pathol, 2000. **114**(1): p. 84-92.
157. Gerber, M.A., S.M. Bahr, and D.H. Gutmann, *Protein 4.1B/differentially expressed in adenocarcinoma of the lung-1 functions as a growth suppressor in meningioma cells by activating Rac1-dependent c-Jun-NH(2)-kinase signaling.* Cancer Res, 2006. **66**(10): p. 5295-303.
158. Gutmann, D.H., et al., *Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas.* Hum Mol Genet, 2000. **9**(10): p. 1495-500.
159. Perry, A., et al., *Merlin, DAL-1, and progesterone receptor expression in clinicopathologic subsets of meningioma: a correlative immunohistochemical study of 175 cases.* J Neuropathol Exp Neurol, 2000. **59**(10): p. 872-9.
160. Yi, C., et al., *Loss of the putative tumor suppressor band 4.1B/Dal1 gene is dispensable for normal development and does not predispose to cancer.* Mol Cell Biol, 2005. **25**(22): p. 10052-9.
161. Nunes, F., et al., *Inactivation patterns of NF2 and DAL-1/4.1B (EPB41L3) in sporadic meningioma.* Cancer Genet Cytogenet, 2005. **162**(2): p. 135-9.
162. Martinez-Glez, V., et al., *Mutational analysis of the DAL-1/4.1B tumour-suppressor gene locus in meningiomas.* Int J Mol Med, 2005. **16**(4): p. 771-4.
163. Kishida, Y., et al., *Epigenetic subclassification of meningiomas based on genome-wide DNA methylation analyses.* Carcinogenesis, 2012. **33**(2): p. 436-41.
164. Di Vinci, A., et al., *HOXA7, 9, and 10 are methylation targets associated with aggressive behavior in meningiomas.* Transl Res, 2012. **160**(5): p. 355-62.
165. Vengoechea, J., et al., *Methylation markers of malignant potential in meningiomas.* J Neurosurg, 2013. **119**(4): p. 899-906.
166. Jun, P., et al., *Epigenetic silencing of the kinase tumor suppressor WNK2 is tumor-type and tumor-grade specific.* Neuro Oncol, 2009. **11**(4): p. 414-22.
167. Reuss, D.E., et al., *Secretory meningiomas are defined by combined KLF4 K409Q and TRAF7 mutations.* Acta Neuropathol, 2013. **125**(3): p. 351-8.
168. Surace, E.I., et al., *Loss of tumor suppressor in lung cancer-1 (TSLC1) expression in meningioma correlates with increased malignancy grade and reduced patient survival.* J Neuropathol Exp Neurol, 2004. **63**(10): p. 1015-27.
169. Schwechheimer, K., L. Zhou, and W. Birchmeier, *E-Cadherin in human brain tumours: loss of immunoreactivity in malignant meningiomas.* Virchows Arch, 1998. **432**(2): p. 163-7.
170. Utsuki, S., et al., *Invasive meningioma is associated with a low expression of E-cadherin and beta-catenin.* Clin Neuropathol, 2005. **24**(1): p. 8-12.
171. Zhou, K., et al., *The potential involvement of E-cadherin and beta-catenins in meningioma.* PLoS One, 2010. **5**(6): p. e11231.
172. Das, A., et al., *Overexpression of mdm2 and p53 and association with progesterone receptor expression in benign meningiomas.* Neuropathology, 2002. **22**(3): p. 194-9.
173. Ragel, B.T. and R.L. Jensen, *Aberrant signaling pathways in meningiomas.* J Neurooncol, 2010. **99**(3): p. 315-24.

174. Johnson, M.D., et al., *Evidence for mitogen-associated protein kinase activation and transduction of mitogenic signals by platelet-derived growth factor in human meningioma cells.* J Neurosurg, 2001. **94**(2): p. 293-300.
175. Yang, S.Y. and G.M. Xu, *Expression of PDGF and its receptor as well as their relationship to proliferating activity and apoptosis of meningiomas in human meningiomas.* J Clin Neurosci, 2001. **8 Suppl 1**: p. 49-53.
176. Schrell, U.M., et al., *Inhibition of proliferation of human cerebral meningioma cells by suramin: effects on cell growth, cell cycle phases, extracellular growth factors, and PDGF-BB autocrine growth loop.* J Neurosurg, 1995. **82**(4): p. 600-7.
177. Carroll, R.S., et al., *Expression and activation of epidermal growth factor receptors in meningiomas.* J Neurosurg, 1997. **87**(2): p. 315-23.
178. Halper, J., et al., *Expression of TGFalpha in meningiomas.* J Neurooncol, 1999. **45**(2): p. 127-34.
179. Johnson, M.D., et al., *The epidermal growth factor receptor is associated with phospholipase C-gamma 1 in meningiomas.* Hum Pathol, 1994. **25**(2): p. 146-53.
180. Wrobel, G., et al., *Microarray-based gene expression profiling of benign, atypical and anaplastic meningiomas identifies novel genes associated with meningioma progression.* Int J Cancer, 2005. **114**(2): p. 249-56.
181. Watson, M.A., et al., *Molecular characterization of human meningiomas by gene expression profiling using high-density oligonucleotide microarrays.* Am J Pathol, 2002. **161**(2): p. 665-72.
182. Nordqvist, A.C. and T. Mathiesen, *Expression of IGF-II, IGFBP-2, -5, and -6 in meningiomas with different brain invasiveness.* J Neurooncol, 2002. **57**(1): p. 19-26.
183. Samoto, K., et al., *Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors.* Cancer Res, 1995. **55**(5): p. 1189-93.
184. Kalkanis, S.N., et al., *Correlation of vascular endothelial growth factor messenger RNA expression with peritumoral vasogenic cerebral edema in meningiomas.* J Neurosurg, 1996. **85**(6): p. 1095-101.
185. Provias, J., et al., *Meningiomas: role of vascular endothelial growth factor/vascular permeability factor in angiogenesis and peritumoral edema.* Neurosurgery, 1997. **40**(5): p. 1016-26.
186. Otsuka, S., et al., *The relationship between peritumoral brain edema and the expression of vascular endothelial growth factor and its receptors in intracranial meningiomas.* J Neurooncol, 2004. **70**(3): p. 349-57.
187. Pistolesi, S., et al., *Meningioma-associated brain oedema: the role of angiogenic factors and pial blood supply.* J Neurooncol, 2002. **60**(2): p. 159-64.
188. Sakuma, T., et al., *Expression of vascular endothelial growth factor-A and mRNA stability factor HuR in human meningiomas.* J Neurooncol, 2008.
189. Kan, P., et al., *Peritumoral edema after stereotactic radiosurgery for intracranial meningiomas and molecular factors that predict its development.* J Neurooncol, 2007. **83**(1): p. 33-8.
190. Jensen, R.L., et al., *Expression of hypoxia inducible factor-1 alpha and correlation with preoperative embolization of meningiomas.* J Neurosurg, 2002. **97**(3): p. 658-67.
191. Bajetto, A., et al., *CXCR4 and SDF1 expression in human meningiomas: a proliferative role in tumoral meningothelial cells in vitro.* Neuro Oncol, 2007. **9**(1): p. 3-11.
192. Johnson, M.D., et al., *Bone morphogenetic protein 4 and its receptors are expressed in the leptomeninges and meningiomas and signal via the Smad pathway.* J Neuropathol Exp Neurol, 2009. **68**(11): p. 1177-83.
193. Johnson, M.D., et al., *Fibroblast growth factor receptor-3 expression in meningiomas with stimulation of proliferation by the phosphoinositide 3 kinase-Akt pathway.* J Neurosurg, 2010. **112**(5): p. 934-9.

194. Johnson, M.D., E. Okediji, and A. Woodard, *Transforming growth factor-beta effects on meningioma cell proliferation and signal transduction pathways.* J Neurooncol, 2004. **66**(1-2): p. 9-16.
195. Johnson, M. and S. Toms, *Mitogenic signal transduction pathways in meningiomas: novel targets for meningioma chemotherapy?* J Neuropathol Exp Neurol, 2005. **64**(12): p. 1029-36.
196. Mawrin, C., et al., *Different activation of mitogen-activated protein kinase and Akt signaling is associated with aggressive phenotype of human meningiomas.* Clin Cancer Res, 2005. **11**(11): p. 4074-82.
197. Johnson, M.D., et al., *Evidence for phosphatidylinositol 3-kinase-Akt-p7S6K pathway activation and transduction of mitogenic signals by platelet-derived growth factor in meningioma cells.* J Neurosurg, 2002. **97**(3): p. 668-75.
198. Jensen, R.L., et al., *In vitro growth inhibition of growth factor-stimulated meningioma cells by calcium channel antagonists.* Neurosurgery, 1995. **36**(2): p. 365-73; discussion 373-4.
199. Jensen, R.L. and R.D. Wurster, *Calcium channel antagonists inhibit growth of subcutaneous xenograft meningiomas in nude mice.* Surg Neurol, 2001. **55**(5): p. 275-83.
200. Kokoglu, E., et al., *Prostaglandin E2 levels in human brain tumor tissues and arachidonic acid levels in the plasma membrane of human brain tumors.* Cancer Lett, 1998. **132**(1-2): p. 17-21.
201. Ragel, B.T., R.L. Jensen, and W.T. Couldwell, *Inflammatory response and meningioma tumorigenesis and the effect of cyclooxygenase-2 inhibitors.* Neurosurg Focus, 2007. **23**(4): p. E7.
202. Kato, Y., et al., *Clinicopathological evaluation of cyclooxygenase-2 expression in meningioma: immunohistochemical analysis of 76 cases of low and high-grade meningioma.* Brain Tumor Pathol, 2014. **31**(1): p. 23-30.
203. James, M.F., et al., *NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth.* Mol Cell Biol, 2009. **29**(15): p. 4250-61.
204. Pachow, D., et al., *mTORC1 inhibitors suppress meningioma growth in mouse models.* Clin Cancer Res, 2013. **19**(5): p. 1180-9.
205. James, M.F., et al., *Regulation of mTOR complex 2 signaling in neurofibromatosis 2-deficient target cell types.* Mol Cancer Res, 2012. **10**(5): p. 649-59.
206. Pecina-Slaus, N., et al., *Genetic and protein changes of E-cadherin in meningiomas.* J Cancer Res Clin Oncol, 2010. **136**(5): p. 695-702.
207. Pecina-Slaus, N., et al., *Meningiomas exhibit loss of heterozygosity of the APC gene.* J Neurooncol, 2008. **87**(1): p. 63-70.
208. Brunner, E.C., et al., *Altered expression of beta-catenin/E-cadherin in meningiomas.* Histopathology, 2006. **49**(2): p. 178-87.
209. Zhou, L., et al., *Merlin-deficient human tumors show loss of contact inhibition and activation of Wnt/beta-catenin signaling linked to the PDGFR/Src and Rac/PAK pathways.* Neoplasia, 2010. **13**(12): p. 1101-12.
210. Ress, A. and K. Moelling, *Bcr is a negative regulator of the Wnt signalling pathway.* EMBO Rep, 2005. **6**(11): p. 1095-100.
211. Cuevas, I.C., et al., *Meningioma transcript profiles reveal deregulated Notch signaling pathway.* Cancer Res, 2005. **65**(12): p. 5070-5.
212. Baia, G.S., et al., *Notch activation is associated with tetraploidy and enhanced chromosomal instability in meningiomas.* Neoplasia, 2008. **10**(6): p. 604-12.
213. Laurendeau, I., et al., *Gene expression profiling of the hedgehog signaling pathway in human meningiomas.* Mol Med, 2010. **16**(7-8): p. 262-70.

214. Varella-Garcia, M., *Molecular cytogenetics in solid tumors: laboratorial tool for diagnosis, prognosis, and therapy.* Oncologist, 2003. **8**(1): p. 45-58.
215. Halling, K.C. and B.R. Kipp, *Fluorescence in situ hybridization in diagnostic cytology.* Hum Pathol, 2007. **38**(8): p. 1137-44.
216. Schneider, B.F., et al., *Loss of chromosomes 22 and 14 in the malignant progression of meningiomas. A comparative study of fluorescence in situ hybridization (FISH) and standard cytogenetic analysis.* Cancer Genet Cytogenet, 1995. **85**(2): p. 101-4.
217. Zattara-Cannoni, H., et al., *Contribution of cytogenetics and FISH in the diagnosis of meningiomas. A study of 189 tumors.* Ann Genet, 1998. **41**(3): p. 164-75.
218. Lopez-Gines, C., et al., *Loss of 1p in recurrent meningiomas. a comparative study in successive recurrences by cytogenetics and fluorescence in situ hybridization.* Cancer Genet Cytogenet, 2001. **125**(2): p. 119-24.
219. Pelz, A.F., et al., *Novel chromosomal aberrations in a recurrent malignant meningioma.* Cancer Genet Cytogenet, 2007. **174**(1): p. 48-53.
220. Maruno, M., et al., *Chromosomal losses and gains in meningiomas: comparative genomic hybridization (CGH) study of the whole genome.* Neurol Res, 1998. **20**(7): p. 612-6.
221. Ozaki, S., et al., *Comparative genomic hybridization analysis of genetic alterations associated with malignant progression of meningioma.* J Neurooncol, 1999. **41**(2): p. 167-74.
222. Woo, K.S., et al., *Characterization of complex chromosome aberrations in a recurrent meningioma combining standard cytogenetic and array comparative genomic hybridization techniques.* Cancer Genet Cytogenet, 2008. **180**(1): p. 56-9.
223. Ketter, R., et al., *Application of oncogenetic trees mixtures as a biostatistical model of the clonal cytogenetic evolution of meningiomas.* Int J Cancer, 2007. **121**(7): p. 1473-80.
224. Sayagues, J.M., et al., *Intratumoral patterns of clonal evolution in meningiomas as defined by multicolor interphase fluorescence in situ hybridization (FISH): is there a relationship between histopathologically benign and atypical/anaplastic lesions?* J Mol Diagn, 2004. **6**(4): p. 316-25.
225. Aarhus, M., M. Lund-Johansen, and P.M. Knappskog, *Gene expression profiling of meningiomas: current status after a decade of microarray-based transcriptomic studies.* Acta Neurochir (Wien), 2011. **153**(3): p. 447-56.
226. Fevre-Montange, M., et al., *Microarray gene expression profiling in meningiomas: differential expression according to grade or histopathological subtype.* Int J Oncol, 2009. **35**(6): p. 1395-407.
227. Stuart, J.E., et al., *Identification of gene markers associated with aggressive meningioma by filtering across multiple sets of gene expression arrays.* J Neuropathol Exp Neurol, 2011. **70**(1): p. 1-12.
228. Aarhus, M., et al., *Microarray analysis reveals down-regulation of the tumour suppressor gene WWOX and up-regulation of the oncogene TYMS in intracranial sporadic meningiomas.* J Neurooncol, 2008. **88**(3): p. 251-9.
229. Fathallah-Shaykh, H.M., et al., *Genomic expression discovery predicts pathways and opposing functions behind phenotypes.* J Biol Chem, 2003. **278**(26): p. 23830-3.
230. Keller, A., et al., *Genome wide expression profiling identifies specific deregulated pathways in meningioma.* Int J Cancer, 2009. **124**(2): p. 346-51.
231. Wang, X., et al., *Analysis of gene expression profiling in meningioma: deregulated signaling pathways associated with meningioma and EGFL6 overexpression in benign meningioma tissue and serum.* PLoS One, 2012. **7**(12): p. e52707.
232. Sayagues, J.M., et al., *Microarray-based analysis of spinal vs. intracranial meningiomas: different clinical, biological, and genetic characteristics associated with distinct patterns of gene expression.* J Neuropathol Exp Neurol, 2006. **65**(5): p. 445-54.

233. Tabernero, M.D., et al., *Patient gender is associated with distinct patterns of chromosomal abnormalities and sex chromosome linked gene-expression profiles in meningiomas.* Oncologist, 2007. **12**(10): p. 1225-36.
234. Carvalho, L.H., et al., *Molecular signatures define two main classes of meningiomas.* Mol Cancer, 2007. **6**(1): p. 64.
235. Holland, H., et al., *High resolution genomic profiling and classical cytogenetics in a group of benign and atypical meningiomas.* Cancer Genet, 2011. **204**(10): p. 541-9.
236. Suva, M.L. and D.N. Louis, *Next-generation molecular genetics of brain tumours.* Curr Opin Neurol, 2013. **26**(6): p. 681-7.
237. Whiteside, T.L., *The tumor microenvironment and its role in promoting tumor growth.* Oncogene, 2008. **27**(45): p. 5904-12.
238. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nat Med, 2013. **19**(11): p. 1423-37.
239. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
240. Pages, F., et al., *In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer.* J Clin Oncol, 2009. **27**(35): p. 5944-51.
241. Mahmoud, S.M., et al., *Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer.* J Clin Oncol, 2011. **29**(15): p. 1949-55.
242. Al-Shibli, K.I., et al., *Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer.* Clin Cancer Res, 2008. **14**(16): p. 5220-7.
243. Mougiakakos, D., et al., *Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma.* Cancer, 2010. **116**(9): p. 2224-33.
244. Tao, H., et al., *Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells.* Lung Cancer, 2012. **75**(1): p. 95-101.
245. Okita, Y., et al., *Role of tumor-infiltrating CD11b+ antigen-presenting cells in the progression of gastric cancer.* J Surg Res, 2014. **186**(1): p. 192-200.
246. Shiao, S.L., et al., *Immune microenvironments in solid tumors: new targets for therapy.* Genes Dev, 2011. **25**(24): p. 2559-72.
247. Mantovani, A., et al., *Tumour immunity: effector response to tumour and role of the microenvironment.* Lancet, 2008. **371**(9614): p. 771-83.
248. Seruga, B., et al., *Cytokines and their relationship to the symptoms and outcome of cancer.* Nat Rev Cancer, 2008. **8**(11): p. 887-99.
249. Lorger, M., *Tumor microenvironment in the brain.* Cancers (Basel), 2012. **4**(1): p. 218-43.
250. Hamilton, A. and N.R. Sibson, *Role of the systemic immune system in brain metastasis.* Mol Cell Neurosci, 2013. **53**: p. 42-51.
251. Wainwright, D.A., et al., *Recent developments on immunotherapy for brain cancer.* Expert Opin Emerg Drugs, 2012. **17**(2): p. 181-202.
252. Riccadonna, C. and P.R. Walker, *Macrophages and Microglia in Brain Malignancies*, in *Tumor Microenvironment and Myelomonocytic Cells*, D.S. Biswas, Editor 2012, InTech. p. 173-200.
253. Johnson, T.S., D.H. Munn, and B.L. Maria, *Modulation of tumor tolerance in primary central nervous system malignancies.* Clin Dev Immunol, 2012. **2012**: p. 937253.
254. Ousman, S.S. and P. Kubes, *Immune surveillance in the central nervous system.* Nat Neurosci, 2012. **15**(8): p. 1096-101.
255. Lossinsky, A.S. and R.R. Shivers, *Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review.* Histol Histopathol, 2004. **19**(2): p. 535-64.

256. Guillemin, G.J. and B.J. Brew, *Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification.* J Leukoc Biol, 2004. **75**(3): p. 388-97.
257. Parney, I.F., J.S. Waldron, and A.T. Parsa, *Flow cytometry and in vitro analysis of human glioma-associated macrophages. Laboratory investigation.* J Neurosurg, 2009. **110**(3): p. 572-82.
258. Streit, W.J., et al., *Role of microglia in the central nervous system's immune response.* Neurol Res, 2005. **27**(7): p. 685-91.
259. Wang, X., et al., *Hypoxia enhances CXCR4 expression favoring microglia migration via HIF-1alpha activation.* Biochem Biophys Res Commun, 2008. **371**(2): p. 283-8.
260. Liang, K.J., et al., *Regulation of dynamic behavior of retinal microglia by CX3CR1 signaling.* Invest Ophthalmol Vis Sci, 2009. **50**(9): p. 4444-51.
261. Kettenmann, H., et al., *Physiology of microglia.* Physiol Rev, 2011. **91**(2): p. 461-553.
262. Yang, I., et al., *The role of microglia in central nervous system immunity and glioma immunology.* J Clin Neurosci, 2010. **17**(1): p. 6-10.
263. Romo-Gonzalez, T., A. Chavarria, and H.J. Perez, *Central nervous system: a modified immune surveillance circuit?* Brain Behav Immun, 2012. **26**(6): p. 823-9.
264. D'Agostino, P.M., et al., *Brain dendritic cells: biology and pathology.* Acta Neuropathol, 2012. **124**(5): p. 599-614.
265. de Graaf, M.T., et al., *Flow cytometric characterization of cerebrospinal fluid cells.* Cytometry B Clin Cytom, 2011. **80**(5): p. 271-81.
266. Svenningsson, A., et al., *Lymphocyte phenotype and subset distribution in normal cerebrospinal fluid.* J Neuroimmunol, 1995. **63**(1): p. 39-46.
267. Rossi, M.L., et al., *Immunohistological study of mononuclear cell infiltrate in malignant gliomas.* Acta Neuropathol, 1987. **74**(3): p. 269-77.
268. Nishie, A., et al., *Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas.* Clin Cancer Res, 1999. **5**(5): p. 1107-13.
269. Roggendorf, W., S. Strupp, and W. Paulus, *Distribution and characterization of microglia/macrophages in human brain tumors.* Acta Neuropathol, 1996. **92**(3): p. 288-93.
270. Strik, H.M., M. Stoll, and R. Meyermann, *Immune cell infiltration of intrinsic and metastatic intracranial tumours.* Anticancer Res, 2004. **24**(1): p. 37-42.
271. Hussain, S.F., et al., *The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses.* Neuro Oncol, 2006. **8**(3): p. 261-79.
272. Rossi, M.L., et al., *Immunocytochemical study of the cellular immune response in meningiomas.* J Clin Pathol, 1988. **41**(3): p. 314-9.
273. Shinonaga, M., et al., *Immunohistological evaluation of macrophage infiltrates in brain tumors. Correlation with peritumoral edema.* J Neurosurg, 1988. **68**(2): p. 259-65.
274. Asai, J., et al., *Fluorescence automatic cell sorter and immunohistochemical investigation of CD68-positive cells in meningioma.* Clin Neurol Neurosurg, 1999. **101**(4): p. 229-34.
275. Mosnier, J.F., et al., *Expression of beta2 integrins and macrophage-associated antigens in meningeal tumours.* Virchows Arch, 2000. **436**(2): p. 131-7.
276. Grund, S., et al., *The microglial/macrophagic response at the tumour-brain border of invasive meningiomas.* Neuropathol Appl Neurobiol, 2009. **35**(1): p. 82-8.
277. Caffo, M., et al., *CD68 and CR3/43 immunohistochemical expression in secretory meningiomas.* Neurosurgery, 2005. **57**(3): p. 551-7; discussion 551-7.
278. Badie, B. and J.M. Schartner, *Flow cytometric characterization of tumor-associated macrophages in experimental gliomas.* Neurosurgery, 2000. **46**(4): p. 957-61; discussion 961-2.
279. Watters, J.J., J.M. Schartner, and B. Badie, *Microglia function in brain tumors.* J Neurosci Res, 2005. **81**(3): p. 447-55.

280. Okada, M., et al., *Tumor-associated macrophage/microglia infiltration in human gliomas is correlated with MCP-3, but not MCP-1.* Int J Oncol, 2009. **34**(6): p. 1621-7.
281. Leung, S.Y., et al., *Monocyte chemoattractant protein-1 expression and macrophage infiltration in gliomas.* Acta Neuropathol, 1997. **93**(5): p. 518-27.
282. Sielska, M., et al., *Distinct roles of CSF family cytokines in macrophage infiltration and activation in glioma progression and injury response.* J Pathol, 2013. **230**(3): p. 310-21.
283. Badie, B., et al., *In vitro modulation of microglia motility by glioma cells is mediated by hepatocyte growth factor/scatter factor.* Neurosurgery, 1999. **44**(5): p. 1077-82; discussion 1082-3.
284. Braun, B., et al., *Expression of G-CSF and GM-CSF in human meningiomas correlates with increased tumor proliferation and vascularization.* J Neurooncol, 2004. **68**(2): p. 131-40.
285. Giometto, B., et al., *Growth factor (M-CSF) and antigenic properties of macrophages in meningioma.* J Neurooncol, 1992. **13**(1): p. 25-33.
286. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-96.
287. Sica, A., P. Allavena, and A. Mantovani, *Cancer related inflammation: the macrophage connection.* Cancer Lett, 2008. **267**(2): p. 204-15.
288. Hagemann, T., et al., *"Re-educating" tumor-associated macrophages by targeting NF-kappaB.* J Exp Med, 2008. **205**(6): p. 1261-8.
289. Pyonteck, S.M., et al., *CSF-1R inhibition alters macrophage polarization and blocks glioma progression.* Nat Med, 2013. **19**(10): p. 1264-72.
290. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. **25**(12): p. 677-86.
291. Nakagawa, J., et al., *TNF expressed by tumor-associated macrophages, but not microglia, can eliminate glioma.* Int J Oncol, 2007. **30**(4): p. 803-11.
292. Hwang, S.Y., et al., *Induction of glioma apoptosis by microglia-secreted molecules: The role of nitric oxide and cathepsin B.* Biochim Biophys Acta, 2009. **1793**(11): p. 1656-68.
293. Galarneau, H., et al., *Increased glioma growth in mice depleted of macrophages.* Cancer Res, 2007. **67**(18): p. 8874-81.
294. Komohara, Y., et al., *Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas.* J Pathol, 2008. **216**(1): p. 15-24.
295. Umemura, N., et al., *Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics.* J Leukoc Biol, 2008. **83**(5): p. 1136-44.
296. Gabrusiewicz, K., et al., *Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas.* PLoS One, 2011. **6**(8): p. e23902.
297. Wu, A., et al., *Glioma cancer stem cells induce immunosuppressive macrophages/microglia.* Neuro Oncol, 2010. **12**(11): p. 1113-25.
298. Hussain, S.F., et al., *Innate immune functions of microglia isolated from human glioma patients.* J Transl Med, 2006. **4**: p. 15.
299. Hussain, S.F., et al., *A novel small molecule inhibitor of signal transducers and activators of transcription 3 reverses immune tolerance in malignant glioma patients.* Cancer Res, 2007. **67**(20): p. 9630-6.
300. Kostianovsky, A.M., et al., *Astrocytic regulation of human monocytic/microglial activation.* J Immunol, 2008. **181**(8): p. 5425-32.
301. Khaled, Y.S., B.J. Ammori, and E. Elkord, *Myeloid-derived suppressor cells in cancer: recent progress and prospects.* Immunol Cell Biol, 2013. **91**(8): p. 493-502.
302. Kohanbash, G. and H. Okada, *Myeloid-derived suppressor cells (MDSCs) in gliomas and glioma-development.* Immunol Invest, 2012. **41**(6-7): p. 658-79.

303. Raychaudhuri, B., et al., *Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma.* Neuro Oncol, 2011. **13**(6): p. 591-9.
304. Rodrigues, J.C., et al., *Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties.* Neuro Oncol, 2010. **12**(4): p. 351-65.
305. Biollaz, G., et al., *Site-specific anti-tumor immunity: differences in DC function, TGF-beta production and numbers of intratumoral Foxp3+ Treg.* Eur J Immunol, 2009. **39**(5): p. 1323-33.
306. Tran Thang, N.N., et al., *Immune infiltration of spontaneous mouse astrocytomas is dominated by immunosuppressive cells from early stages of tumor development.* Cancer Res, 2010. **70**(12): p. 4829-39.
307. Gousias, K., et al., *Phenotypical analysis, relation to malignancy and prognostic relevance of ICOS+T regulatory and dendritic cells in patients with gliomas.* J Neuroimmunol, 2013. **264**(1-2): p. 84-90.
308. Tyrinova, T.V., et al., *Cytotoxic activity of ex-vivo generated IFNalpha-induced monocyte-derived dendritic cells in brain glioma patients.* Cell Immunol, 2013. **284**(1-2): p. 146-53.
309. Fang, L., et al., *The immune cell infiltrate populating meningiomas is composed of mature, antigen-experienced T and B cells.* Neuro Oncol, 2013. **15**(11): p. 1479-90.
310. Roessler, K., W. Dietrich, and K. Kitz, *Expression of BCL-2 oncoprotein on tumor cells and tumor-infiltrating lymphocytes (TIL) in meningiomas.* Neurosurg Rev, 1999. **22**(4): p. 205-9.
311. Becker, I. and W. Roggendorf, *Immunohistological investigation of mononuclear cell infiltrates in meningiomas.* Acta Neuropathol, 1989. **79**(2): p. 211-6.
312. Farmer, J.P., et al., *Characterization of lymphoid cells isolated from human gliomas.* J Neurosurg, 1989. **71**(4): p. 528-33.
313. Yu, J.S., et al., *Intratumoral T cell subset ratios and Fas ligand expression on brain tumor endothelium.* J Neurooncol, 2003. **64**(1-2): p. 55-61.
314. Saito, T., et al., *Immunohistochemical analysis of tumor-infiltrating lymphocytes and major histocompatibility antigens in human gliomas and metastatic brain tumors.* Surg Neurol, 1988. **29**(6): p. 435-42.
315. Stevens, A., I. Kloter, and W. Roggendorf, *Inflammatory infiltrates and natural killer cell presence in human brain tumors.* Cancer, 1988. **61**(4): p. 738-43.
316. Dunn, G.P., I.F. Dunn, and W.T. Curry, *Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma.* Cancer Immun, 2007. **7**: p. 12.
317. Lohr, J., et al., *Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta.* Clin Cancer Res, 2011. **17**(13): p. 4296-308.
318. Kim, Y.H., et al., *Tumour-infiltrating T-cell subpopulations in glioblastomas.* Br J Neurosurg, 2012. **26**(1): p. 21-7.
319. Yang, I., et al., *CD8+ T-cell infiltrate in newly diagnosed glioblastoma is associated with long-term survival.* J Clin Neurosci, 2010. **17**(11): p. 1381-5.
320. Kmiecik, J., et al., *Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level.* J Neuroimmunol, 2013. **264**(1-2): p. 71-83.
321. Roussel, E., et al., *Predominance of a type 2 intratumoural immune response in fresh tumour-infiltrating lymphocytes from human gliomas.* Clin Exp Immunol, 1996. **105**(2): p. 344-52.
322. Hao, C., et al., *Cytokine and cytokine receptor mRNA expression in human glioblastomas: evidence of Th1, Th2 and Th3 cytokine dysregulation.* Acta Neuropathol, 2002. **103**(2): p. 171-8.

323. Li, G., et al., *Expression and switching of TH1/TH2 type cytokines gene in human gliomas.* Chin Med Sci J, 2005. **20**(4): p. 268-72.
324. Zisakis, A., et al., *Comparative analysis of peripheral and localised cytokine secretion in glioblastoma patients.* Cytokine, 2007. **39**(2): p. 99-105.
325. Kumar, R., et al., *Th1/Th2 cytokine imbalance in meningioma, anaplastic astrocytoma and glioblastoma multiforme patients.* Oncol Rep, 2006. **15**(6): p. 1513-6.
326. Shimato, S., et al., *Profound tumor-specific Th2 bias in patients with malignant glioma.* BMC Cancer, 2012. **12**: p. 561.
327. Jacobs, J.F., et al., *Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors.* Neuro Oncol, 2009. **11**(4): p. 394-402.
328. El Andaloussi, A. and M.S. Lesniak, *An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme.* Neuro Oncol, 2006. **8**(3): p. 234-43.
329. Fecci, P.E., et al., *Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma.* Cancer Res, 2006. **66**(6): p. 3294-302.
330. El Andaloussi, A. and M.S. Lesniak, *CD4+ CD25+ FoxP3+ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas.* J Neurooncol, 2007. **83**(2): p. 145-52.
331. El Andaloussi, A., Y. Han, and M.S. Lesniak, *Prolongation of survival following depletion of CD4+CD25+ regulatory T cells in mice with experimental brain tumors.* J Neurosurg, 2006. **105**(3): p. 430-7.
332. Mitchell, D.A., et al., *Monoclonal antibody blockade of IL-2 receptor alpha during lymphopenia selectively depletes regulatory T cells in mice and humans.* Blood, 2011. **118**(11): p. 3003-12.
333. Maes, W., et al., *Depletion of regulatory T cells in a mouse experimental glioma model through anti-CD25 treatment results in the infiltration of non-immunosuppressive myeloid cells in the brain.* Clin Dev Immunol, 2013. **2013**: p. 952469.
334. Jarry, U., et al., *Treg depletion followed by intracerebral CpG-ODN injection induce brain tumor rejection.* J Neuroimmunol, 2014. **267**(1-2): p. 35-42.
335. Waldhauer, I. and A. Steinle, *NK cells and cancer immunosurveillance.* Oncogene, 2008. **27**(45): p. 5932-43.
336. Kmiecik, J., J. Zimmer, and M. Chekenya, *Natural killer cells in intracranial neoplasms: presence and therapeutic efficacy against brain tumours.* J Neurooncol, 2014. **116**(1): p. 1-9.
337. Castriconi, R., et al., *NK cells recognize and kill human glioblastoma cells with stem cell-like properties.* J Immunol, 2009. **182**(6): p. 3530-9.
338. Avril, T., et al., *Human glioblastoma stem-like cells are more sensitive to allogeneic NK and T cell-mediated killing compared with serum-cultured glioblastoma cells.* Brain Pathol, 2012. **22**(2): p. 159-74.
339. Poli, A., et al., *Targeting glioblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival.* Oncotarget, 2013. **4**(9): p. 1527-46.
340. Alizadeh, D., et al., *Induction of anti-glioma natural killer cell response following multiple low-dose intracerebral CpG therapy.* Clin Cancer Res, 2010. **16**(13): p. 3399-408.
341. Friese, M.A., et al., *RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo.* Cancer Res, 2004. **64**(20): p. 7596-603.
342. Pellegatta, S., et al., *The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates.* Oncoimmunology, 2013. **2**(3): p. e23401.

343. Vaquero, J., et al., *Natural killer cells in meningiomas.* Histol Histopathol, 1991. **6**(3): p. 369-72.
344. Vaquero, J., et al., *Presence and significance of NK cells in glioblastomas.* J Neurosurg, 1989. **70**(5): p. 728-31.
345. Vaquero, J., et al., *Immunohistochemical study of IOT-10 natural killer cells in brain metastases.* Acta Neurochir (Wien), 1990. **104**(1-2): p. 17-20.
346. Yang, I., et al., *Immune cell infiltrate differences in pilocytic astrocytoma and glioblastoma: evidence of distinct immunological microenvironments that reflect tumor biology.* J Neurosurg, 2011. **115**(3): p. 505-11.
347. Crane, C.A., et al., *TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients.* Neuro Oncol, 2010. **12**(1): p. 7-13.
348. Bo, L., S.J. Mork, and H. Nyland, *An immunohistochemical study of mononuclear cells in meningiomas.* Neuropathol Appl Neurobiol, 1992. **18**(6): p. 548-58.
349. Epari, S., et al., *Chordoid meningioma, an uncommon variant of meningioma: a clinicopathologic study of 12 cases.* J Neurooncol, 2006. **78**(3): p. 263-9.
350. Hewedi, I.H., et al., *Perspectives on the immunologic microenvironment of astrocytomas.* Cancer Manag Res, 2013. **5**: p. 293-9.
351. Yasuda, K., et al., *Detection of lymphocytes in malignant gliomas by monoclonal antibodies.* J Neurol Neurosurg Psychiatry, 1983. **46**(8): p. 734-7.
352. Nelson, B.H., *CD20+ B cells: the other tumor-infiltrating lymphocytes.* J Immunol, 2010. **185**(9): p. 4977-82.
353. Candolfi, M., et al., *B cells are critical to T-cell-mediated antitumor immunity induced by a combined immune-stimulatory/conditionally cytotoxic therapy for glioblastoma.* Neoplasia, 2011. **13**(10): p. 947-60.
354. Inoue, S., et al., *Inhibitory effects of B cells on antitumor immunity.* Cancer Res, 2006. **66**(15): p. 7741-7.
355. Pillai, R., S. Kannan, and G.J. Chandran, *The immunohistochemistry of solid tumours: potential problems for new laboratories.* Natl Med J India, 1993. **6**(2): p. 71-5.
356. Bloch, W., Y. Korkmaz, and D. Steinritz, *Immunohistochemistry for Structural and Functional Analysis in Cardiovascular Research*, in *Practical Methods in Cardiovascular Research* S. Dhein, F.W. Mohr, and M. Delmar, Editors. 2005, Springer Berlin Heidelberg. p. 457-484.
357. Legres, L.G., et al., *Beyond laser microdissection technology: follow the yellow brick road for cancer research.* Am J Cancer Res, 2014. **4**(1): p. 1-28.
358. Virgo, P.F. and G.J. Gibbs, *Flow cytometry in clinical pathology.* Ann Clin Biochem, 2012. **49**(Pt 1): p. 17-28.
359. Davidson, B., et al., *The diagnostic and research applications of flow cytometry in cytopathology.* Diagn Cytopathol, 2012. **40**(6): p. 525-35.
360. Chang, Q. and D. Hedley, *Emerging applications of flow cytometry in solid tumor biology.* Methods, 2012. **57**(3): p. 359-67.
361. Robinson, J., *Flow Cytometry*, in *Encyclopedia of Biomaterials and Biomedical Engineering*, W. G.E. and B. G.L., Editors. 2004, Marcel Dekker, Inc. . p. 630-40
362. Corver, W.E. and C.J. Cornelisse, *Flow cytometry of human solid tumours: clinical and research applications.* Current Diagnostic Pathology, 2002. **8**(4): p. 249-67.
363. Alexiou, G.A., et al., *DNA content is associated with malignancy of intracranial neoplasms.* Clin Neurol Neurosurg, 2013. **115**(9): p. 1784-7.
364. Zellner, A., et al., *DNA ploidy and cell-cycle analysis in intracranial meningiomas and hemangiopericytomas: a study with high-resolution DNA flow cytometry.* Int J Cancer, 1998. **79**(2): p. 116-20.
365. Myong, N.H. and J.G. Chi, *Correlation of histopathologic classification with proliferative activity and DNA ploidy in 120 intracranial meningiomas, with special reference to atypical meningioma.* J Korean Med Sci, 1997. **12**(3): p. 221-7.

366. Schartner, J.M., et al., *Impaired capacity for upregulation of MHC class II in tumor-associated microglia.* Glia, 2005. **51**(4): p. 279-85.
367. Bloch, O., et al., *Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages.* Clin Cancer Res, 2013.
368. Badie, B., et al., *Expression of Fas ligand by microglia: possible role in glioma immune evasion.* J Neuroimmunol, 2001. **120**(1-2): p. 19-24.
369. Badie, B., et al., *Dexamethasone-induced abolition of the inflammatory response in an experimental glioma model: a flow cytometry study.* J Neurosurg, 2000. **93**(4): p. 634-9.
370. Waziri, A., et al., *Preferential in situ CD4+CD56+ T cell activation and expansion within human glioblastoma.* J Immunol, 2008. **180**(11): p. 7673-80.
371. Prestegarden, L., et al., *Glioma cell populations grouped by different cell type markers drive brain tumor growth.* Cancer Res, 2010. **70**(11): p. 4274-9.
372. Ogden, A.T., et al., *Identification of A2B5+CD133- tumor-initiating cells in adult human gliomas.* Neurosurgery, 2008. **62**(2): p. 505-14; discussion 514-5.
373. Rebetz, J., et al., *Glial progenitor-like phenotype in low-grade glioma and enhanced CD133-expression and neuronal lineage differentiation potential in high-grade glioma.* PLoS One, 2008. **3**(4): p. e1936.
374. Balik, V., et al., *Flow cytometry analysis of neural differentiation markers expression in human glioblastomas may predict their response to chemotherapy.* Cell Mol Neurobiol, 2009. **29**(6-7): p. 845-58.
375. Rooprai, H.K., et al., *CD44 expression in human meningiomas: An immunocytochemical, immunohistochemical and flow cytometric analysis.* Int J Oncol, 1999. **14**(5): p. 855-60.
376. Rath, P., et al., *Isolation and characterization of a population of stem-like progenitor cells from an atypical meningioma.* Exp Mol Pathol, 2011. **90**(2): p. 179-88.
377. Paz-Bouza, J.I., et al., *Transrectal fine needle aspiration biopsy of the prostate combining cytomorphologic, DNA ploidy status and cell cycle distribution studies.* Pathol Res Pract, 1994. **190**(7): p. 682-9.
378. Cruz, I., et al., *Evaluation of multiparameter flow cytometry for the detection of breast cancer tumor cells in blood samples.* Am J Clin Pathol, 2005. **123**(1): p. 66-74.
379. S. Matarraz, C.F., M. Albors, C.Teodosio, A. López, M. Jara-Acevedo,C. Cervero, G. Caballero, O. Gutierrez, A. Orfao., *Cell-cycle distributionof different cell compartments in normalvs. reactive bone marrow: A frame of reference for the study ofdysplastic hematopoiesis.* Cytometry B Clin Cytom, 2011. **80**(6): p. 354-61.
380. Matarraz, S., et al., *Cell-cycle distribution of different cell compartments in normal vs. reactive bone marrow: a frame of reference for the study of dysplastic hematopoiesis.* Cytometry B Clin Cytom, 2011. **80**(6): p. 354-61.
381. Almeida, J., et al., *Comparative analysis of the morphological, cytochemical, immunophenotypical, and functional characteristics of normal human peripheral blood lineage(-)/CD16(+)/HLA-DR(+)/CD14(-/lo) cells, CD14(+) monocytes, and CD16(-) dendritic cells.* Clin Immunol, 2001. **100**(3): p. 325-38.
382. Martin-Martin, L., et al., *Immunophenotypical, morphologic, and functional characterization of maturation-associated plasmacytoid dendritic cell subsets in normal adult human bone marrow.* Transfusion, 2009. **49**(8): p. 1692-1708.
383. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.
384. Ohara, N., et al., *An immunohistochemical study on HLA-DR expression in human meningiomas.* Acta Neuropathol, 1992. **84**(1): p. 110-2.
385. Dirkx, A.E., et al., *Monocyte/macrophage infiltration in tumors: modulators of angiogenesis.* J Leukoc Biol, 2006. **80**(6): p. 1183-96.
386. Dietrich, D., et al., *Analysis of DNA methylation of multiple genes in microdissected cells from formalin-fixed and paraffin-embedded tissues.* J Histochem Cytochem, 2009. **57**(5): p. 477-89.

387. Biggerstaff, J., et al., *Enumeration of leukocyte infiltration in solid tumors by confocal laser scanning microscopy.* BMC Immunol, 2006. **7**: p. 16.
388. Curran, S., et al., *Laser capture microscopy.* Mol Pathol, 2000. **53**(2): p. 64-8.
389. Fuller, A.P., et al., *Laser capture microdissection and advanced molecular analysis of human breast cancer.* J Mammary Gland Biol Neoplasia, 2003. **8**(3): p. 335-45.
390. Sluka, P., et al., *Application of laser-capture microdissection to analysis of gene expression in the testis.* Prog Histochem Cytochem, 2008. **42**(4): p. 173-201.
391. Hallam, S., et al., *Activated macrophages in the tumour microenvironment-dancing to the tune of TLR and NF-kappaB.* J Pathol, 2009. **219**(2): p. 143-52.
392. Van Hal, P.T., et al., *Regulation of aminopeptidase-N (CD13) and Fc epsilon RIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages.* J Immunol, 1992. **149**(4): p. 1395-401.
393. Pfister, M., et al., *NAD degradation and regulation of CD38 expression by human monocytes/macrophages.* Eur J Biochem, 2001. **268**(21): p. 5601-8.
394. Messmer, U.K., U.K. Reed, and B. Brune, *Bcl-2 protects macrophages from nitric oxide-induced apoptosis.* J Biol Chem, 1996. **271**(33): p. 20192-7.
395. Marzio, R., J. Mauel, and S. Betz-Corradin, *CD69 and regulation of the immune function.* Immunopharmacol Immunotoxicol, 1999. **21**(3): p. 565-82.
396. Cheng, Y.X., et al., *CD2 identifies a monocyte subpopulation with immunoglobulin E-dependent, high-level expression of Fc epsilon RI.* Clin Exp Allergy, 2006. **36**(11): p. 1436-45.
397. Cui, W., et al., *The intracellular domain of CD44 promotes the fusion of macrophages.* Blood, 2006. **107**(2): p. 796-805.
398. Wang, J.H., et al., *Structure of a heterophilic adhesion complex between the human CD2 and CD58 (LFA-3) counterreceptors.* Cell, 1999. **97**(6): p. 791-803.
399. Schenkel, A.R., et al., *CD99 plays a major role in the migration of monocytes through endothelial junctions.* Nat Immunol, 2002. **3**(2): p. 143-50.
400. Veenbergen, S. and A.B. van Spriel, *Tetraspanins in the immune response against cancer.* Immunol Lett, 2011. **138**(2): p. 129-36.
401. Erreni, M., A. Mantovani, and P. Allavena, *Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer.* Cancer Microenviron, 2011. **4**(2): p. 141-54.
402. Laoui, D., et al., *Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions.* Int J Dev Biol, 2011. **55**(7-9): p. 861-7.
403. Qian, B.Z. and J.W. Pollard, *Macrophage diversity enhances tumor progression and metastasis.* Cell, 2010. **141**(1): p. 39-51.
404. Buddingh, E.P., et al., *Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents.* Clin Cancer Res, 2011. **17**(8): p. 2110-9.
405. Ma, J., et al., *The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time.* BMC Cancer, 2010. **10**: p. 112.
406. Thompson, E.D., et al., *Tumor masses support naive T cell infiltration, activation, and differentiation into effectors.* J Exp Med, 2010. **207**(8): p. 1791-804.
407. Knutson, K.L., M.L. Disis, and L.G. Salazar, *CD4 regulatory T cells in human cancer pathogenesis.* Cancer Immunol Immunother, 2007. **56**(3): p. 271-85.
408. Chen, G., et al., *Discordant protein and mRNA expression in lung adenocarcinomas.* Mol Cell Proteomics, 2002. **1**(4): p. 304-13.
409. Celis, J.E., et al., *Gene expression profiling: monitoring transcription and translation products using DNA microarrays and proteomics.* FEBS Lett, 2000. **480**(1): p. 2-16.
410. Maier, T., M. Guell, and L. Serrano, *Correlation of mRNA and protein in complex biological samples.* FEBS Lett, 2009. **583**(24): p. 3966-73.
411. Wiley, H.S., *Trafficking of the ErbB receptors and its influence on signaling.* Exp Cell Res, 2003. **284**(1): p. 78-88.

412. Grange, C., et al., *Phenotypic characterization and functional analysis of human tumor immune infiltration after mechanical and enzymatic disaggregation.* J Immunol Methods, 2011. **372**(1-2): p. 119-26.
413. Smeets, A.W., et al., *Comparison of tissue disaggregation techniques of transitional cell bladder carcinomas for flow cytometry and chromosomal analysis.* Cytometry, 1987. **8**(1): p. 14-9.
414. Mina-Osorio, P., *The moonlighting enzyme CD13: old and new functions to target.* Trends Mol Med, 2008. **14**(8): p. 361-71.
415. Mazzocca, A., F. Liotta, and V. Carloni, *Tetraspanin CD81-regulated cell motility plays a critical role in intrahepatic metastasis of hepatocellular carcinoma.* Gastroenterology, 2008. **135**(1): p. 244-256.
416. Kawashima, M., et al., *CD9 expression in solid non-neuroepithelial tumors and infiltrative astrocytic tumors.* J Histochem Cytochem, 2002. **50**(9): p. 1195-203.
417. Funakoshi, T., et al., *Expression of tetraspanins in human lung cancer cells: frequent downregulation of CD9 and its contribution to cell motility in small cell lung cancer.* Oncogene, 2003. **22**(5): p. 674-87.
418. Ovalle, S., et al., *The tetraspanin CD9 inhibits the proliferation and tumorigenicity of human colon carcinoma cells.* Int J Cancer, 2007. **121**(10): p. 2140-52.
419. Mawrin, C., et al., *Reduced Activity of CD13/Aminopeptidase N (APN) in Aggressive Meningiomas Is Associated with Increased Levels of SPARC.* Brain Pathol, 2010. **20**(1): p. 200-10.
420. Panagopoulos, A.T., et al., *Expression of cell adhesion proteins and proteins related to angiogenesis and fatty acid metabolism in benign, atypical, and anaplastic meningiomas.* J Neurooncol, 2008. **89**(1): p. 73-87.
421. Lewy-Trenda, I., et al., *CD44 expression in human meningiomas: an immunohistochemical analysis.* Pol J Pathol, 2004. **55**(1): p. 33-7.
422. Figarella-Branger, D., et al., *Cell-adhesion molecules in human meningiomas: correlation with clinical and morphological data.* Neuropathol Appl Neurobiol, 1997. **23**(2): p. 113-22.
423. Mosnier, J.F., et al., *Expression of the bcl-2 oncoprotein in meningiomas.* Am J Clin Pathol, 1996. **106**(5): p. 652-9.
424. Wernicke, A.G., et al., *Assessment of Epidermal Growth Factor Receptor (EGFR) expression in human meningioma.* Radiat Oncol, 2010. **5**: p. 46.
425. Smith, J.S., et al., *Association between absence of epidermal growth factor receptor immunoreactivity and poor prognosis in patients with atypical meningioma.* J Neurosurg, 2007. **106**(6): p. 1034-40.
426. Lichtor, T., M.A. Kurpakus, and M.E. Gurney, *Expression of insulin-like growth factors and their receptors in human meningiomas.* J Neurooncol, 1993. **17**(3): p. 183-90.
427. Abdelzaher, E., et al., *Recurrence of benign meningiomas: predictive value of proliferative index, BCL2, p53, hormonal receptors and HER2 expression.* Br J Neurosurg, 2011. **25**(6): p. 707-13.
428. Loussouarn, D., et al., *Prognostic value of HER2 expression in meningiomas: an immunohistochemical and fluorescence in situ hybridization study.* Hum Pathol, 2006. **37**(4): p. 415-21.
429. Shinoura, N., et al., *RNA expression of complement regulatory proteins in human brain tumors.* Cancer Lett, 1994. **86**(2): p. 143-9.
430. Kim, D.D. and W.C. Song, *Membrane complement regulatory proteins.* Clin Immunol, 2006. **118**(2-3): p. 127-36.
431. Chang, C.C., M. Campoli, and S. Ferrone, *HLA class I defects in malignant lesions: what have we learned?* Keio J Med, 2003. **52**(4): p. 220-9.

432. Oldford, S.A., et al., *Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients.* Int Immunol, 2006. **18**(11): p. 1591-602.
433. Matsushita, K., et al., *Strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients: Possible involvement of c-myc suppression by interferon-gamma in situ.* Cancer Sci, 2006. **97**(1): p. 57-63.
434. Rangel, L.B., et al., *Anomalous expression of the HLA-DR alpha and beta chains in ovarian and other cancers.* Cancer Biol Ther, 2004. **3**(10): p. 1021-7.
435. Reid, P.A. and C. Watts, *Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments.* Nature, 1990. **346**(6285): p. 655-7.
436. Watanabe, A., et al., *CD14-mediated signal pathway of Porphyromonas gingivalis lipopolysaccharide in human gingival fibroblasts.* Infect Immun, 1996. **64**(11): p. 4488-94.
437. Liu, S., et al., *Expression of CD14 by hepatocytes: upregulation by cytokines during endotoxemia.* Infect Immun, 1998. **66**(11): p. 5089-98.
438. Summers, K.L., et al., *Monocyte-macrophage antigen expression on chondrocytes.* J Rheumatol, 1995. **22**(7): p. 1326-34.
439. Fearns, C., et al., *Murine CD14 gene expression in vivo: extramyeloid synthesis and regulation by lipopolysaccharide.* J Exp Med, 1995. **181**(3): p. 857-66.
440. Xia, Y., K. Yamagata, and T.L. Krukoff, *Differential expression of the CD14/TLR4 complex and inflammatory signaling molecules following i.c.v. administration of LPS.* Brain Res, 2006. **1095**(1): p. 85-95.
441. Chakravarty, S. and M. Herkenham, *Toll-like receptor 4 on nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines.* J Neurosci, 2005. **25**(7): p. 1788-96.
442. Shabo, I., et al., *Expression of the macrophage antigen CD163 in rectal cancer cells is associated with early local recurrence and reduced survival time.* Int J Cancer, 2009. **125**(8): p. 1826-31.
443. Kalala, J.P., J. Caemaert, and L. De Ridder, *Primary resected meningiomas: relapses and proliferation markers.* In Vivo, 2004. **18**(4): p. 411-6.
444. Swartz, M.A., et al., *Tumor microenvironment complexity: emerging roles in cancer therapy.* Cancer Res, 2012. **72**(10): p. 2473-80.
445. Domingues, P.H., et al., *Immunophenotypic identification and characterization of tumor cells and infiltrating cell populations in meningiomas.* Am J Pathol, 2012. **181**(5): p. 1749-61.
446. Jochems, C. and J. Schlom, *Tumor-infiltrating immune cells and prognosis: the potential link between conventional cancer therapy and immunity.* Exp Biol Med (Maywood). 2011. **236**(5): p. 567-79.
447. Erdag, G., et al., *Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma.* Cancer Res, 2012. **72**(5): p. 1070-80.
448. Montelli Tde, C., et al., *Genetic and modifying factors that determine the risk of brain tumors.* Cent Nerv Syst Agents Med Chem, 2011. **11**(1): p. 8-30.
449. Ishizaki, S., et al., *Role of CD69 in acute lung injury.* Life Sci, 2012. **90**(17-18): p. 657-65.
450. Pfistershammer, K., et al., *CD63 as an activation-linked T cell costimulatory element.* J Immunol, 2004. **173**(10): p. 6000-8.
451. Frankenberger, M., et al., *Immunologic characterization of normal human pleural macrophages.* Am J Respir Cell Mol Biol, 2000. **23**(3): p. 419-26.
452. Andreesen, R., et al., *Surface phenotype analysis of human monocyte to macrophage maturation.* J Leukoc Biol, 1990. **47**(6): p. 490-7.

453. Wang, Z.Q., et al., *Interleukin-10 induces macrophage apoptosis and expression of CD16 (FcgammaRIII) whose engagement blocks the cell death programme and facilitates differentiation.* Immunology, 2001. **102**(3): p. 331-7.
454. Pure, E. and C.A. Cuff, *A crucial role for CD44 in inflammation.* Trends Mol Med, 2001. **7**(5): p. 213-21.
455. Duff, M.D., et al., *Analysis of gene expression in the tumor-associated macrophage.* J Surg Res, 2007. **142**(1): p. 119-28.
456. Kaji, K., et al., *Functional association of CD9 with the Fc gamma receptors in macrophages.* J Immunol, 2001. **166**(5): p. 3256-65.
457. Helm, O., et al., *Tumor-associated macrophages exhibit pro- and anti-inflammatory properties by which they impact on pancreatic tumorigenesis.* Int J Cancer, 2014.
458. Ino, Y., et al., *Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer.* Br J Cancer, 2013. **108**(4): p. 914-23.
459. Geldhof, A.B., et al., *Antagonistic effect of NK cells on alternatively activated monocytes: a contribution of NK cells to CTL generation.* Blood, 2002. **100**(12): p. 4049-58.
460. Krausgruber, T., et al., *IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses.* Nat Immunol, 2011. **12**(3): p. 231-8.
461. Bowdridge, S. and W.C. Gause, *Regulation of alternative macrophage activation by chromatin remodeling.* Nat Immunol, 2010. **11**(10): p. 879-81.
462. Predina, J., et al., *Changes in the local tumor microenvironment in recurrent cancers may explain the failure of vaccines after surgery.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. E415-24.
463. Calandra, T. and T. Roger, *Macrophage migration inhibitory factor: a regulator of innate immunity.* Nat Rev Immunol, 2003. **3**(10): p. 791-800.
464. Mitchell, R.A., *Mechanisms and effectors of MIF-dependent promotion of tumourigenesis.* Cell Signal, 2004. **16**(1): p. 13-9.
465. Rendon, B.E., et al., *Mechanisms of macrophage migration inhibitory factor (MIF)-dependent tumor microenvironmental adaptation.* Exp Mol Pathol, 2009. **86**(3): p. 180-5.
466. Kamimura, A., et al., *Intracellular distribution of macrophage migration inhibitory factor predicts the prognosis of patients with adenocarcinoma of the lung.* Cancer, 2000. **89**(2): p. 334-41.
467. Muramaki, M., et al., *Clinical utility of serum macrophage migration inhibitory factor in men with prostate cancer as a novel biomarker of detection and disease progression.* Oncol Rep, 2006. **15**(1): p. 253-7.
468. Fersching, D.M., et al., *Apoptosis-related biomarkers sFAS, MIF, ICAM-1 and PAI-1 in serum of breast cancer patients undergoing neoadjuvant chemotherapy.* Anticancer Res, 2012. **32**(5): p. 2047-58.
469. Zheng, Y.X., et al., *CD74 and macrophage migration inhibitory factor as therapeutic targets in gastric cancer.* World J Gastroenterol, 2012. **18**(18): p. 2253-61.
470. Wang, X.B., et al., *Elevated expression of macrophage migration inhibitory factor correlates with tumor recurrence and poor prognosis of patients with gliomas.* J Neurooncol, 2012. **106**(1): p. 43-51.
471. Domingues, P.H., et al., *Proposal for a new risk stratification classification for meningioma based on patient age, WHO tumor grade, size, localization, and karyotype.* Neuro Oncol, 2014: p. [Epub ahead of print].
472. Rushmere, N.K., et al., *Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer.* Int J Cancer, 2004. **108**(6): p. 930-6.

473. Ravindranath, N.M. and C. Shuler, *Cell-surface density of complement restriction factors (CD46, CD55, and CD59): oral squamous cell carcinoma vs. other solid tumors.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2007. **103**(2): p. 231-9.
474. Spendlove, I., et al., *Complement decay accelerating factor (DAF)/CD55 in cancer.* Cancer Immunol Immunother, 2006. **55**(8): p. 987-95.
475. Shamah, S.M., et al., *Detection of activated platelet-derived growth factor receptors in human meningioma.* Cancer Res, 1997. **57**(18): p. 4141-7.
476. Tyagi, D., et al., *Expression of Bcl2 proto-oncogene in primary tumors of the central nervous system.* Neurol India, 2002. **50**(3): p. 290-4.
477. Funa, K. and M. Sasahara, *The Roles of PDGF in Development and During Neurogenesis in the Normal and Diseased Nervous System.* J Neuroimmune Pharmacol, 2013.
478. Doucette, T., et al., *Bcl-2 promotes malignant progression in a PDGF-B-dependent murine model of oligodendroglioma.* Int J Cancer, 2011. **129**(9): p. 2093-103.
479. Rempel, S.A., S. Ge, and J.A. Gutierrez, *SPARC: a potential diagnostic marker of invasive meningiomas.* Clin Cancer Res, 1999. **5**(2): p. 237-41.
480. Hemler, M.E., *Tetraspanin functions and associated microdomains.* Nat Rev Mol Cell Biol, 2005. **6**(10): p. 801-11.
481. Hirano, C., et al., *Tetraspanin gene expression levels as potential biomarkers for malignancy of gingival squamous cell carcinoma.* Int J Cancer, 2009. **124**(12): p. 2911-6.
482. Sorkin, A., *Internalization of the epidermal growth factor receptor: role in signalling.* Biochem Soc Trans, 2001. **29**(Pt 4): p. 480-4.
483. Laurendeau, I., et al., *Gene expression profiling of ErbB receptors and ligands in human meningiomas.* Cancer Invest, 2009. **27**(6): p. 691-8.
484. Wickremesekera, A., C.M. Hovens, and A.H. Kaye, *Expression of ErbB-1 and ErbB-2 in meningioma.* J Clin Neurosci, 2010. **17**(9): p. 1155-8.
485. Mahzouni, P. and M. Movahedipour, *An immunohistochemical study of HER2 expression in meningioma and its correlation with tumor grade.* Pathol Res Pract, 2012. **208**(4): p. 221-4.
486. Lagaudriere-Gesbert, C., et al., *Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity.* Cell Immunol, 1997. **182**(2): p. 105-12.
487. Zumkeller, W. and M. Westphal, *The IGF/IGFBP system in CNS malignancy.* Mol Pathol, 2001. **54**(4): p. 227-9.
488. Wick, M.R., *Immunohistochemical approaches to the diagnosis of undifferentiated malignant tumors.* Ann Diagn Pathol, 2008. **12**(1): p. 72-84.
489. Ishizawa, K., et al., *Olig2 and CD99 are useful negative markers for the diagnosis of brain tumors.* Clin Neuropathol, 2008. **27**(3): p. 118-28.
490. Renshaw, A.A., *O13 (CD99) in spindle cell tumors: reactivity with hemangiopericytoma, solitary fibrous tumor, synovial sarcoma, and meningioma but rarely with sarcomatoid mesothelioma.* App Immunohistoch, 1995. **3**: p. 250-6.
491. Vaz, R., et al., *Cerebral edema associated with meningiomas: the role of peritumoral brain tissue.* J Neurooncol, 1998. **36**(3): p. 285-91.
492. Arismendi-Morillo, G. and A. Castellano, *Tumoral micro-blood vessels and vascular microenvironment in human astrocytic tumors. A transmission electron microscopy study.* J Neurooncol, 2005. **73**(3): p. 211-7.
493. Brown, R.C. and T.P. Davis, *Calcium modulation of adherens and tight junction function: a potential mechanism for blood-brain barrier disruption after stroke.* Stroke, 2002. **33**(6): p. 1706-11.
494. Perry, A., et al., *Meningioma grading: an analysis of histologic parameters.* Am J Surg Pathol, 1997. **21**(12): p. 1455-65.
495. Urbschat, S., et al., *Clonal cytogenetic progression within intratumorally heterogeneous meningiomas predicts tumor recurrence.* Int J Oncol, 2011. **39**(6): p. 1601-8.

496. Ketter, R., et al., *Correspondence of tumor localization with tumor recurrence and cytogenetic progression in meningiomas.* Neurosurgery, 2008. **62**(1): p. 61-9.
497. Pfisterer, W.K., et al., *Implicating chromosomal aberrations with meningioma growth and recurrence: results from FISH and MIB-I analysis of grades I and II meningioma tissue.* J Neurooncol, 2008. **87**(1): p. 43-50.
498. Pfisterer, W.K., et al., *Diagnostic and prognostic significance of genetic regional heterogeneity in meningiomas.* Neuro Oncol, 2004. **6**(4): p. 290-9.
499. Cahill, K.S. and E.B. Claus, *Treatment and survival of patients with nonmalignant intracranial meningioma: results from the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute. Clinical article.* J Neurosurg, 2011. **115**(2): p. 259-67.
500. Kane, A.J., et al., *Anatomic location is a risk factor for atypical and malignant meningiomas.* Cancer, 2011. **117**(6): p. 1272-8.
501. Pfisterer, W.K., et al., *Fluorescent in situ hybridization and ex vivo 1H magnetic resonance spectroscopic examinations of meningioma tumor tissue: is it possible to identify a clinically-aggressive subset of benign meningiomas?* Neurosurgery, 2007. **61**(5): p. 1048-59.
502. Barbera, S., et al., *Genetic changes with prognostic value in histologically benign meningiomas.* Clin Neuropathol, 2013. **32**(4): p. 311-7.
503. van Alkemade, H., et al., *Impaired survival and long-term neurological problems in benign meningioma.* Neuro Oncol, 2012. **14**(5): p. 658-66.
504. Ildan, F., et al., *Predicting the probability of meningioma recurrence in the preoperative and early postoperative period: a multivariate analysis in the midterm follow-up.* Skull Base, 2007. **17**(3): p. 157-71.
505. Ko, K.W., et al., *Relationship between malignant subtypes of meningioma and clinical outcome.* J Clin Neurosci, 2007. **14**(8): p. 747-53.
506. Palma, L., et al., *Long-term prognosis for atypical and malignant meningiomas: a study of 71 surgical cases.* J Neurosurg, 1997. **86**(5): p. 793-800.
507. Ayerbe, J., et al., *Risk factors predicting recurrence in patients operated on for intracranial meningioma. A multivariate analysis.* Acta Neurochir (Wien), 1999. **141**(9): p. 921-32.
508. Vranic, A., et al., *Mitotic count, brain invasion, and location are independent predictors of recurrence-free survival in primary atypical and malignant meningiomas: a study of 86 patients.* Neurosurgery, 2010. **67**(4): p. 1124-32.
509. Huang, Q., et al., *Increased co-expression of macrophage migration inhibitory factor and matrix metalloproteinase 9 is associated with tumor recurrence of meningioma.* Int J Med Sci, 2013. **10**(3): p. 276-85.

Supplementary data

Supplementary Table 1. Levels of tumor-infiltrating lymphocyte (TIL) subpopulations, with particular focus on regulatory T cells identified by $CD4^{+}CD25^{high}CD127^{-/low}$ cells, and infiltrating tissue macrophages (TiMa) expressing the CD206 M2 phenotype-associated marker in a series of 33 meningioma samples.

	ID	%TIL *	%B cells #	%NK cells #	%TCD8 #	%TCD4 #	%Treg #	No. Tregs per 10^{-5}	%TiMa *	TiMa CD206 (MFI)	%TiMa $CD206^{+}$ #	%TiMa $CD206^{+}$ *
	76	20	2.6	10	60	27	0.3	58	40	83	15	6
	77 R	6	1	28	39	33	1.2	52	43	544	30	13
	78	4	1.9	6	65	27	0.4	13	32	1008	60	19
	82	0.4	0	14	70	17	0.2	1	15	164	0	0
	83 R	12	5.7	4	58	32	0.4	52	42	1142	60	25
	84	44	0.8	4	46	49	3.6	1318	17	571	50	9
Complex karyotype	91 R	0.2	1.6	18	22	59	1.4	2	3	1590	50	2
	92	2	0	8	63	29	0.02	0	13	107	7	1
	93 R	5.5	0	5	42	53	0	0	2	203	35	1
	99	0.8	2.2	13	41	43	2.1	16	7	74	0	0
	100	2.8	19	5	41	36	0.3	9	11	260	2	0
	101	3.3	4	8	46	43	0.3	4	16	415	25	4
	104 &	10	0	4	72.5	23.5	3	242	60	275	20	12
	105 &	0.7	25	25	35	15	0.1	1	12	348	20	2
		8 ± 12	5 ± 8	11 ± 8	50 ± 15	35 ± 13	0.95 ± 1.2	126 ± 349	22 ± 18	485 ± 457	27 ± 22	7 ± 8
	89	1	2.6	35	24	38	1	6	20	143	10	2
	90	0.8	1.3	32	43	23	0.4	2	13	111	10	1
Monosomy22 / del(22q)	94	5	0.8	29	41	30	0.9	25	50	54	5	3
	96	2	0.7	27	54	18	0.1	2	54	524	30	16
	97	0.5	3.2	15	42	40	2.7	10	30	167	10	3
		2 ± 2	2 ± 1	28 ± 8	41 ± 11	30 ± 10	1 ± 1	9 ± 10	33 ± 18	200 ± 186	13 ± 10	5 ± 6
	79	4.5	2.1	2	58	38	0.2	6	19	173	10	2
	80	0.5	4.7	7	44	45	0.5	2	15	501	50	8
	81	1.6	0.3	9	75	17	0.4	5	4	684	40	2
	85	1	25	5	24	46	3.4	38	5	90	0	0
	86	11	3.4	24	28	44	0.2	0	38	187	15	6
	87	5	0.2	5	64	31	0.1	5	22	154	10	2
	88 R	15	4.2	2	56	37	2.7	367	15	143	20	3
Diploid	95	0.25	1.8	24	45	30	0.75	2	5	540	15	1
	98	0.3	1.4	39	33	26	2.9	12	6	425	50	3
	102	1.6	0	0	80	20	0	0	0	0	0	0
	103	0.9	0	5	62	33	3	23	10	551	25	3
	106 &	0.05							4	477	19	1
	107 &	0.5	1.5	11.5	12	75	0	0	6	4487	50	3
	108 &	9	3	7	60	30	0.4	26	8	34	1	0
		4 ± 5	4 ± 7	11 ± 11	49 ± 20	36 ± 15	1.1 ± 1.3	37 ± 100	11 ± 10	603 ± 1139	22 ± 19	2 ± 2

MFI, mean fluorescence intensity;
TIL, tumor-infiltrating lymphocytes;
Results are expressed as the percentage of positive cells for: (*) the whole tumor, or (#) a specific population (TIL or TiMa);
& Case also stained for CD163;
R Recurrent tumor.

Supplementary Table 2. Immunophenotypic characterization of meningeal neoplastic cells evaluated by multiparameter flow cytometry: percentage of positive cases, positive cells per case and amount of expression - mean fluorescence intensity (MFI) for each analyzed marker (n=51).

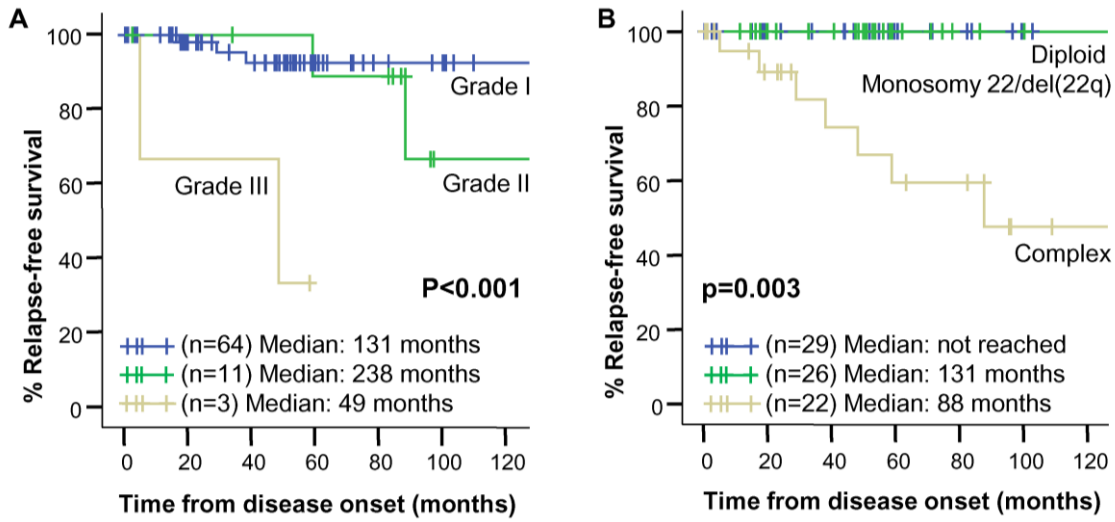
Marker	N. of positive samples / Total samples (%)	% of positive cells *	Mean amount of protein (MFI)/tumour cell *
CD9	51/51 (100)	97±10	26278±17530
CD44	51/51 (100)	80±26	6415±9288
CD55	51/51 (100)	96±12	5083±2321
CD59	51/51 (100)	99±2	26146±13792
CD63	51/51 (100)	90±15	3528±1648
CD81	51/51 (100)	98±5	17464±14372
HLA-I	51/51 (100)	90±14	11159±9199
CD13	51/51 (100)	89±15	18985±24534
PDGFRβ	49/51 (96)	77±28	2294±2273
HER2/neu	49/51 (96)	73±26	1933±1692
IGFR	49/51 (96)	73±25	1272±935
CD38	44/51 (86)	66±27	1849±2015
Bcl2	44/51 (86)	65±24	915±584
HLA-DR	41/51 (80)	69±23	2640±2900
EGFR	40/51(78)	69±24	768±707
CD14	39/51 (76)	76±19	872±864
CD53	32/51 (63)	62±24	553±848
CD58	30/51 (59)	61±22	359±332
CD99	24/51 (47)	60±21	287±241
CD2	11/51 (22)	57±24	354±266

* Results expressed as mean ± one standard deviation (SD)

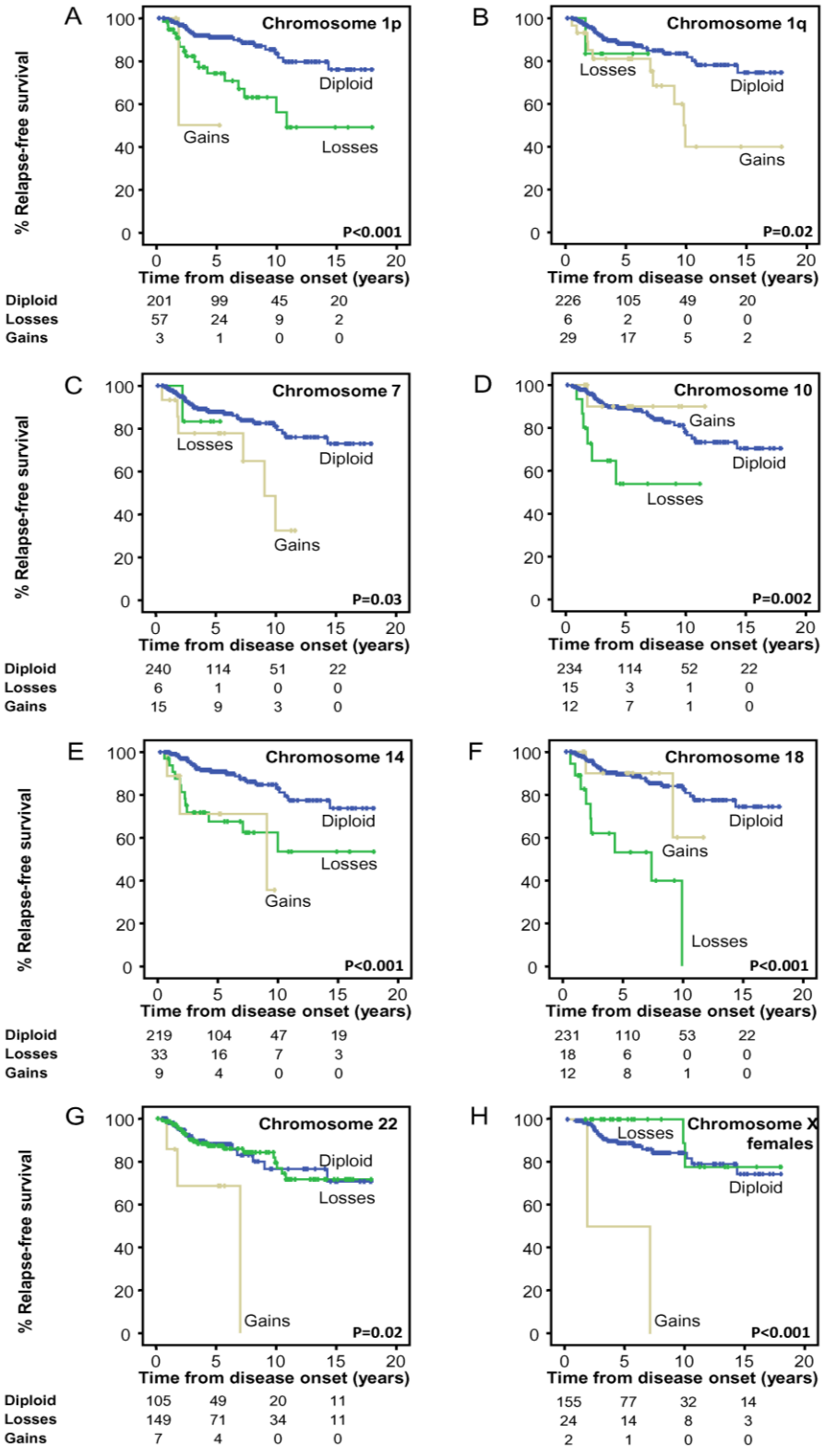
Supplementary Table 3. Association between the cytogenetic alterations found for individual chromosomes by iFISH and tumor grade (n=302), incidence of relapse and relapse-free survival (RFS) of meningioma patients (n=261).

Variables		Patient distribution	Tumor WHO grade (WHO)			P-value	No. of recurrences	p-value	% of patients relapse-free			75% RFS (months)	Univariate analysis (P-value)	Multivariate analysis #	
			I	II	III				5y-RFS *	10y-RFS *	15y-RFS *			P-value	HR (95% CI)
Chromosome 1p	diploid	235 (78%)	216	12	7		23 (10%)		91% ± 2%	84% ± 4%	76% ± 6%	NR			
	losses	64 (21%)	47	16	1	<0.001	18 (28%)	0.001	74% ± 7%	63% ± 8%	49% ± 11%	51	<0.001		
	gains	3 (1%)	1	2	0		1 (33%)		50% ± 35%	-	-	22			
Chromosome 1q	diploid	265 (88%)	239	19	7		30 (11%)		88% ± 2%	84% ± 3%	75% ± 5%	172			
	losses	6 (2%)	4	2	0	0.001	1 (17%)	0.001	80% ± 18%	-	-	NR	0.01		
	gains	31 (10%)	21	9	1		11 (36%)		81% ± 8%	50% ± 14%	40% ± 14%	88			
Chromosome 7	diploid	280 (93%)	250	23	7		35 (13%)		88% ± 2%	81% ± 4%	73% ± 5%	172			
	losses	7 (2%)	7	0	0	<0.001	1 (14%)	0.01	83% ± 15%	-	-	NR	0.03	0.05	
	gains	15 (5%)	7	7	1		6 (40%)		78% ± 11%	47% ± 18%	-	88			4 (1-10)
Chromosome 9	diploid	285 (95%)	254	24	7		36 (13%)		88% ± 2%	80% ± 4%	72% ± 5%	172			
	losses	4 (1%)	4	0	0	<0.001	1 (25%)	0.03	100% ± 0%	0% ± 0%	-	119	N.S.		
	gains	13 (4%)	6	6	1		5 (39%)		67% ± 14%	67% ± 14%	45% ± 20%	29			
Chromosome 10	diploid	271 (90%)	242	23	6		35 (13%)		89% ± 2%	80% ± 4%	70% ± 5%	127			
	losses	19 (6%)	14	4	1	N.S.	6 (32%)	N.S.	54% ± 15%	54% ± 15%	-	23	0.002		
	gains	12 (4%)	8	3	1		1 (8%)		90% ± 10%	90% ± 10%	-	NR			
Chromosome 14	diploid	255 (84%)	233	16	6		26 (10%)		91% ± 2%	83% ± 4%	74% ± 6%	172			
	losses	38 (13%)	26	11	1	<0.001	13 (34%)	<0.001	68% ± 9%	62% ± 9%	54% ± 12%	29	<0.001	0.001	3 (2-7)
	gains	9 (3%)	5	3	1		3 (33%)		71% ± 18%	36% ± 27%	-	22			7 (2-28)
Chromosome 15	diploid	282 (93%)	253	23	6		39 (14%)		87% ± 2%	79% ± 4%	70% ± 5%	127			
	losses	5 (2%)	2	2	1	<0.001	0 (0%)	N.S.	100% ± 0%	-	-	-	N.S.		
	gains	15 (5%)	9	5	1		3 (20%)		86% ± 10%	57% ± 24%	-	109			
Chromosome 17	diploid	278 (92%)	252	20	6		37 (13%)		87% ± 3%	80% ± 4%	71% ± 5%	131			
	losses	3 (1%)	1	2	0	<0.001	0 (0%)	N.S.	100% ± 0%	100% ± 0%	-	-	N.S.		
	gains	21 (7%)	11	8	2		5 (24%)		89% ± 7%	61% ± 17%	46% ± 19%	109			
Chromosome 18	diploid	270 (89%)	245	19	6		31 (12%)	<0.001	90% ± 2%	84% ± 3%	74% ± 5%	172			
	losses	20 (7%)	13	6	1	<0.001	9 (45%)		53% ± 13%	0% ± 0%	0% ± 0%	27	<0.001	<0.001	5 (2-13)
	gains	12 (4%)	6	5	1		2 (17%)		90% ± 10%	60% ± 25%	-	109			
Chromosome 22	diploid	129 (43%)	115	10	4		15 (12%)		88% ± 4%	77% ± 6%	71% ± 8%	172			
	losses	166 (55%)	147	16	3	<0.001	24 (15%)	N.S.	87% ± 3%	82% ± 4%	72% ± 6%	127	0.02		
	gains	7 (2%)	2	4	1		3 (43%)		69% ± 19%	0% ± 0%	0% ± 0%	22			
Chromosome X	diploid	183 (87%)	171	11	1		20 (11%)		89% ± 3%	84% ± 4%	75% ± 7%	172			
females	losses	26 (12%)	21	4	1	<0.001	2 (8%)	<0.001	100% ± 0%	89% ± 11%	78% ± 14%	NR	<0.001		
	gains	2 (1%)	0	1	1		2 (100%)		50% ± 35%	0% ± 0%	0% ± 0%	22			
males	diploid	89 (98%)	71	13	5	N.S.	18 (20%)	N.S.	80% ± 5%	68% ± 8%	62% ± 9%	98	N.S.		
	gains	2 (2%)	1	1	0		0 (0%)		100% ± 0%	-	-	-			
Chromosome Y	normal	65 (72%)	54	8	3		14 (22%)		80% ± 6%	65% ± 9%	57% ± 11%	98			
	losses	24 (26%)	17	5	2	N.S.	4 (17%)	N.S.	79% ± 9%	79% ± 9%	79% ± 9%	NR	N.S.		
	gains	2 (2%)	1	1	0		0 (0%)		100% ± 0%	-	-	-			

Results expressed as number of cases and percentage between brackets or as * percentage of cases ± SE (standard error); NS: statistically not significant (p>0.05); NR: not reached. HR: Hazard ratio; CI: confidence interval; # the good prognosis category was selected as the reference group.



Supplementary Figure 1. Impact of tumour WHO grade and cytogenetics on relapse-free survival of meningioma patients. Relapse-free survival curves of meningioma patients classified according to tumour grade (n=78; panel A) and cytogenetics (n=77; panel B) are shown.



Supplementary Figure 2. Relapse-free survival (RFS) of meningioma patients (n=261) grouped according to the iFISH alterations detected for individual chromosomes. Only relapse-free survival curves of meningioma patients grouped according to the status (normal, lost and gained) of those chromosomes analyzed by iFISH which showed a significant prognostic impact for RFS, are shown. RFS curves for chromosomes 1p, 1q, 7, 10, 14, 18, 22 and X in females are plotted in panels A to H, respectively.