Exosomal MicroRNA Signatures in Central Nervous System Diseases

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A thesis submitted to fulfil the requirements
for the degree of Doctor of Philosophy

Abstract

During the last decade there has been a growing interest in studying extracellular vesicles, in particular exosomes and their miRNA contents. Exosomes are released by almost all cell types. They are packed with specific information, stable against degradation processes, are small and flexible enough to cross the blood-brain barrier (BBB), and are readily found in biological fluids including blood. MicroRNAs (miRNAs) are involved in nearly every cellular process and play a regulatory role in central nervous system (CNS) associated diseases. Accordingly, exosomal miRNAs could be ideal biomarkers to measure CNS disease activity and treatment response.

In this thesis, the aim was to establish a robust protocol to investigate whether the differential expression of serum exosomal miRNA can be used as a biomarker for the accurate diagnosis of the CNS diseases multiple sclerosis (MS) and glioblastoma multiforme (GBM), as well as for the monitoring of disease progression and treatment response.

Exosomes were purified from serum and their RNA contents profiled using high-throughput sequencing. In my first study, I profiled exosome–associated miRNAs in serum samples from MS patients and identified distinct biomarkers for the diagnosis of MS and identification of the disease subtype. In my second study, I investigated the effect of treatment in MS patients. I hypothesised that the deregulation of serum exosomal miRNAs is associated with the efficacy of therapy and is predictive of MS activity phases. Finally, I studied serum exosomal miRNA profiles to discover diagnostic biomarkers for GBM, and to demonstrate the applicability of my protocol to other neurological diseases.

Taken together, my results demonstrate the exceptional utility of serum exosomal miRNA profiles as a blood-based biomarker to diagnose the CNS associated diseases, using a robust and easily reproducible protocol.

Acknowledgements

The preparation of this thesis would not have been possible without the love and support of many people around me. For that, I am grateful.

To my supervisor Associate Professor Michael Buckland, thank you for giving me the opportunity to join your lab as a PhD student, and for your optimism, support and generous guidance.

To Associate Professor Catherine Suter, thank you for your generosity, knowledge and unwavering scientific vision, without which this project would never have come to fruition.

To my co-supervisor, Dr Fatemeh Vafaee, thank you for your insightful advice and constructive criticism which has helped me throughout.

To my co-supervisor Professor Michael Barnett, thank you for accepting me and giving me access to your clinic and clinical trial samples.

To Dr Kimberley Kaufman, thank you for your support and friendship during this PhD candidature and for bringing brightness to our scientific and social life at the Brain and Mind Centre (BMC). Thank you for giving me the opportunity to contribute to your researches.

I also have Dr Damian Holsinger to thank for accepting me as a researcher in his group, introducing me to Associate Professor Michael Buckland when I was searching for a PhD project, and helping me to achieve Australian Postgraduate Award Scholarship.

Throughout this candidature, I have had the honour of working alongside many brilliant scientists at the Victor Chang Research Institute (VCRI) and BMC. I would like to thank all members of the Suter Laboratory family at VCRI, especially Paul Young and Navind Jayasooriah for helping me to understand bioinformatics and Dr Jennifer Cropley for her great patience and generosity sharing her skills and solving bench problems. Thanks also Suzy, Matthias, Alastair and Sally. To Susannah at the BMC, thanks for your mass spectrometric results and all the GBM

samples. To Grace and Maggie, thank you with your help to collect and process blood samples.

And thank you to David Edmonds for reading this thesis from a non-biomedical background.

And a special thanks to the participating patients who willingly provided consent to be included in this study, and to the staff and nurses at the Multiple Sclerosis clinic at the BMC who helped me with sample collection.

Finally, I would like to express my deepest gratitude to my husband, Mohsen, and my lovely daughter, Avin, who have inspired me to follow my passion and who have supported me wholeheartedly. I am grateful to my mum and dad who encouraged me in my moments of doubt and patiently tolerated my absence. All of my academic achievements are a credit to my parents. I also wish to express my heartfelt appreciation to my gorgeous sister and wonderful brothers who have always been there and make me feel blessed.

Contents

List of abbreviations

AB	apoptotic body
AUC	area under the ROC curve
BBB	blood-brain barrier
cDNA	complementary DNA
CI	confidence intervals
CNS	central nervous system
CSF	cerebrospinal fluid
DMT	disease modifying therapy
EBV	Epstein Barr virus
EDSS	expanded disability status scale
ESCRT	endosomal-sorting complex required for transport
EVs	extracellular vesicle
GBM	glioblastoma multiforme
Gd	gadolinium
Gd-MRI	gadolinium-based contrast agents for MRI scans
GEO	Gene Expression Omnibus
GO	gene ontology
HC	healthy control
HGG	high-grade glioma (grades III and IV)
HLA	human leukocyte antigen
IDH	isocitrate dehydrogenase isoforms
IDH^{MUT}	IDH-mutant
IDH^{WT}	IDH-wildtype
ILV	intraluminal vesicle
LGG	low-grade glioma (grades I and II)
LOO-CV	leave-one-out cross validation
LR	logistic regression
MGMT	O^6-Methylguanine-DNA-Methyltransferase
miRNA	microRNA
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
MSigDB	Molecular Signatures Database
MV	microvesicle
MVB	multivesicular bodies
NGS	next-generation sequencing
NTA	nanoparticle tracking analysis

OOB	out-of-bag
PPMS	primary progressive multiple sclerosis
pre-miRNA	precursor miRNAs
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homolog
RF	random forest
ROC	receiver operator characteristic
RRMS	relapsing-remitting multiple sclerosis
rRNA	ribosomal RNA
S1P	sphingosine 1-phosphate
SEC	size-exclusive chromatography
siRNA	small interfering RNA
sncRNA	small non-coding RNA
SPMS	secondary progressive multiple sclerosis
TCGA	The Cancer Genome Atlas
TEM	transmission electron microscopy
TP53	tumor protein 53
tRNA	transfer RNA
UC	ultracentrifugation
WB	Western blotting
WHO	World Health Organization

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Publications and presentations arising from work in this thesis

Ebrahimkhani S, Vafaee F, Young P, Hur S, Hawke S, Devenney E, Beadnall H, Barnett MH, Suter CM, Buckland ME. *Exosomal microRNA signatures in multiple sclerosis reflect disease status.* Scientific Reports, 7: 14293 | DOI:10.1038/s41598-017-14301-3.

S.E. performed technical work including exosome purification, small RNA sequencing, bioinformatics and manuscript preparation. F.V. and P.Y. performed bioinformatics analysis. S.S.J. Hur provided technical assistance, data interpretation and manuscript preparation. S.H., E.D., H.B. and M.H.B. assisted with clinical sample and data collection and provided critical review of the manuscript. C.M.S. assisted with small RNA sequencing protocols, data interpretation and manuscript preparation. M.E.B. provided overall experimental design and data interpretation, and assisted with manuscript preparation.

The first author contributed over 80% of the work to this manuscript.

Ebrahimkhani S*, Beadnall H*, Chenyu Wang C, Suter C, Barnett MH, Buckland M & Vafaee F. Characterising the effect of fingolimod therapy on exosomal miRNAs in patients with relapsing multiple sclerosis. (In final preparation for submission to Neurology) (*These Authors equally contributed to the manuscript)

MHB, HNB, and MEB conceptualized and designed the study and provided critical review of the manuscript. HNB collected patients' specimens, clinical and MRI information. SE and CW were involved in technical work for data acquisition; SE performed exosome purification and RNA extraction. FV developed the machine learning and bioinformatics pipelines. FV and SE analysed data and generated the results. All authors were involved in data interpretation. FV, SE and CMS drafted the manuscript and prepared the figures. All authors revised and approved the final version.

Shared co-authorship was granted as HB performed crucial clinical assessments on the patient cohort. The candidate (SE) contributed ~80%.

Ebrahimkhani S, Vafaee F, Hallal S, Wei H, Lee M, Young P, Seelan L, Shivalingam B, Beadnall H, Barnett MH, Suter C, Buckland M and Kaufman K. Deep sequencing of circulating exosomal microRNA allows non-invasive glioblastoma diagnosis. (Under review: npj Precision Oncology - Nature)

All authors contributed to manuscript preparation and approved the submission of the work presented here. Specific contributions are as follows: S.E. performed technical work, including serum processing, exosome purification, electron microscopy, small RNA sequencing, data analysis, and manuscript preparation. F.V. and P.Y. developed and performed the bioinformatics analytical pipeline. S.H. performed technical work, including serum processing, NanoSight particle tracking, and mass spectrometry. H.W and M.Y.T.L characterized clinical cases, including molecular characterizations of tumour tissue. L.S and B.S assisted with clinical sample procurement and case characterization. H.B and M.H.B provided clinical characterizations of multiple sclerosis cases. C.M.S. assisted with small RNA sequencing protocols and data interpretation. M.E.B. provided experimental design and data interpretation. K.L.K provided experimental design, cohort characterization, proteomics methods, bioinformatics, data interpretation, and presentation. M.E.B and K.L.K are guarantors of this work.

The first author contributed over 80% of the work to this manuscript.

Mallawaaratchy DM, Hallal S, Ly L, **Ebrahimkhani S**, Wei H, Christopherson RI, Buckland ME and Kaufman KL (2016) *Comprehensive proteome profiling of glioblastoma-derived extracellular vesicles provide insight into tumour cell invasion.* Neuro-Oncology 131 (2); 233-244.

Hallal S, Mallawaaratchy D, Wei H, **Ebrahimkhani S**, Stringer B, Day B, Boyd A, Guillemin G, Buckland M and Kaufman K. Extracellular vesicles from glioblastoma cells stimulate normal astrocytes to acquire an invasive phenotype via p53 inhibition and induction of MYC. (Under review: Molecular Neurobiology)

Oral presentations

Serum exosomal miRNAs as potential biomarkers in the central nervous system, Progress in MS Research Conference, Sydney, October 2017

Exosomal microRNA signatures in multiple sclerosis reflect disease status, International Extracellular Vesicles (ISEV) Conference, Canada, 2017 (with a poster presentation: accepted)

Monitoring standardised treatment efficacy of multiple sclerosis in molecular level, ISEV Conference, Toronto, Canada, May 2017 (with a poster presentation: accepted)

An optimised protocol to purify and profile serum exosomal small non-coding RNA, Bosch Young Investigator Committee, Sydney University, 2016

Serum exosomal small noncoding RNAs as potential biomarkers in multiple sclerosis, NSW Inter-university Neuroscience and Mental Health Conference, Sydney, 2016

Biomarker discovery for multiple sclerosis, 24th Australian Society of Medical Research (ASMR) NSW Annual Scientific Meeting, Sydney, 2016

Deep sequencing of circulating exosomal miRNA as biomarker in central nervous system, Bioinformatics Research Symposium, Sydney, 2016 (fast forward talk and poster presentation)

Poster presentations

Deep sequencing of circulating exosomal miRNA allows non-invasive glioblastoma diagnosis, 10th COGNO Annual Scientific Meeting, October 2017

Serum exosomal RNA: a biomarker for multiple sclerosis, Brain and Mind Symposium, Sydney University, October 2017

Circulating small non-coding RNAs expression as potential biomarker in multiple sclerosis progression, St. Vincent's Hospital Symposium, September 2017.

Serum exosomes in glioblastoma patients: an opportunity for non-invasive diagnosis and monitoring, Sydney Catalyst International Translational Cancer Research Symposium, 2017.

An optimised protocol to profile EV small RNA from human and mouse serum, Australian Extracellular Vesicles Conference, Mantra Lorne, Victoria, 2017.

An optimised protocol to purify free circulating and exosomal RNA,17th International Victor Chang Symposium, Sydney, 2016.

Profiling circulating small non-coding RNAs as potential biomarker in the central nervous system progression, Second EMBL PhD symposium. EMBL Australia, Melbourne, 2015.

An optimised protocol to purify free circulating and exosomal RNA, 23rd St. Vincent's Campus Research Symposium, 2015.

Chapter 1:

Introduction and literature review

Introduction

Biomarkers are molecules that reveal biological state and may indicate the presence of a disease or dysfunction. There is growing interest in finding biomarkers to aid the diagnosis and prognosis of central nervous system (CNS) diseases.[1] One of the most substantial barriers to finding biomarkers for CNS conditions is the presence of the blood-brain barrier (BBB). The brain is firmly protected by a highly selective semipermeable membrane that separates circulating blood from the extracellular fluid in the CNS. This barrier has made the brain's chemistry difficult to monitor. However, the discovery of the roles of free circulating microRNA (miRNA) in biology over the last decade offers hope that these molecules may provide effective biomarkers for CNS conditions.[2]

miRNAs are small (18–25 nucleotides), non-coding RNA molecules that regulate gene expression post-transcriptionally.[3] A single miRNA can target multiple genes, and an individual mRNA may be regulated by distinct miRNAs.[4] miRNAs have been shown to be involved in the regulation of many molecular signalling pathways affecting various cellular processes.[5,6] It has been reported that miRNAs are abundant in the brain, and deregulation of their function has been implicated in human diseases.[7]

Exosomes contain a selective package of small regulatory RNA and are enriched in miRNAs.[7-9] Exosomes are membrane–bound vesicles, small enough to cross the BBB[8,9] and released by almost all cell types. In many neurologic and inflammatory diseases there is a significant increase in circulating exosome concentration.[7-9]

This project aims to develop a robust protocol to purify serum exosomal miRNAs using unbiased next-generation sequencing to identify and validate their potential as biomarkers of disease activity and therapeutic efficacy in multiple sclerosis and glioblastoma, and to predict the functional role of dysregulated miRNAs using integrative bioinformatics.

Extracellular vesicles

Extracellular vesicles (EVs) are small vesicles (30 nm to 1 μm) enclosed by a phospholipid bilayer. They are released by almost all cell types and are readily found in biological fluids including blood.[10,11] When EVs were first identified in the 20th century, their roles were not fully appreciated and they were dismissed by many as biochemical artifacts.[12] However, EVs are now widely accepted as an essential component in fundamental cellular responses including intercellular communication and immune reactions. Along with other attributes, their relatively stable structure safeguards their contents against degradation processes.[13,14] Therefore, there is a growing interest in the use of the RNA contents of EVs as biomarkers.

Discovery of EVs

Chargaff and West initially reported cell-derived vesicles in 1946 as a pro-coagulant particle in platelet-free plasma. They centrifuged plasma at different speeds then measured the clotting time. It was recorded that prolonged high-speed centrifugation extended the clotting time of the supernatant, which indicated the presence of subcellular clotting particles.[11] In 1967, Peter Wolf identified the subcellular particles by electron microscopy as small lipid-rich vesicles (20–50 nm), originating from platelets, which he named ‘platelet dust’.[15] One decade later, it was reported that fetal calf serum also contained numerous microvesicles (30–60 nm).[11] Then, in the mid-1980s, Johnstone observed that immature sheep reticulocytes release small membrane–enclosed vesicles, termed ‘exosomes’, to the conditioned culture medium during their maturation into erythrocytes.[14] Released exosomes had compatible characteristics with the plasma membrane of reticulocytes. It had been earlier observed that mature erythrocytes reduce their enzyme activity compared to reticulocytes. Therefore, it was believed that these vesicles function only to remove unnecessary proteins.[12,14] Two years later, in 1989, the same group reported that although the plasma membrane of reticulocytes contains both lysosomal activity and transferrin receptor proteins, released vesicles carried transferrin receptor as an abundant membrane protein but expressed no lysosomal activity. This observation suggested a highly selective pathway for protein sorting in exosomes.[16]

Extracellular vesicles have since been purified from most body fluids. They play a role in cell-to-cell communication in both normal physiological processes and the pathology underlying several diseases.[12,17]

Subtypes of EVs

Early studies revealed different types of vesicle, but did not use an unified terminology. Now, according to their size, origin, and mode of secretion (biogenesis), EVs are grouped into three main classes: apoptotic bodies (ABs), microvesicles (MVs), and exosomes. These are produced by different cell types in different conditions *(Figure 1-1)*.[18-20]

Apoptotic bodies are 800–5000 nm in diameter and unlike MVs and exosomes, which are released from normal viable cells, ABs are generated from the fragmentation of the cell membrane of apoptotic cells *(Figure 1-1A)*.[21,22] This study does not examine ABs.

Microvesicles are 100–1000 nm in size, heterogeneous in shape, and are directly shed from the cell membrane *(Figure 1-1B)*. The biogenesis of MVs is the result of interaction between lipids and proteins. Vesicle formation is induced by phospholipid redistribution complemented by calcium-dependent enzymes like flippase and floppase. Contractions of cytoskeletal structures complete the budding process.[19,23]

Exosomes are the smallest vesicles, with a size of 30–150 nm and a characteristic well-delimited round morphology when observed under transmission electron microscopy.[11] They are released to the extracellular environment after formation of intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) in the cell interior, transport of MVBs to the plasma membrane and fusion of MVBs with the plasma membrane *(Figure 1-1C)*.[24]

Although the formation and release mechanisms of MVs and exosomes are different the size of large exosomes and small MVs are comparable.[25] Despite efforts to define the distinction between various subsets of EVs, no consensus has developed. Currently the International Society of Extracellular Vesicles recommends using the generic term ‘EVs’ for all vesicles isolated from body fluids by currently available methods.[26]

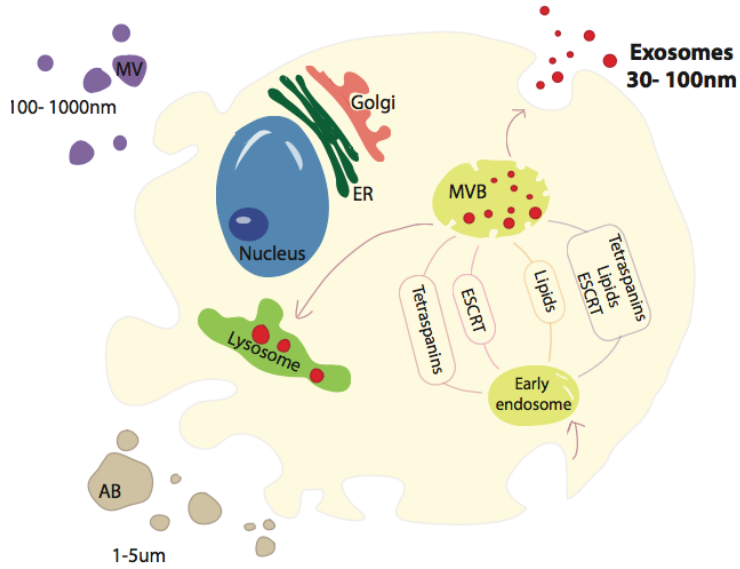


Figure 1-1 Extracellular vesicles

A) Apoptotic bodies are released from cells undergoing death. B) Microvesicles form by outward blebbing of the plasma membrane with incorporation of cytosolic proteins. C) Exosomes form intracellularly, fuse to the plasma membrane and release to the extracellular space.

Exosome biogenesis

Exosomes originate from endocytic pathways so as a result contain endosome-associated proteins such as annexins and flotillin. Some of these proteins (e.g., Alix and Tsg101) are required in exosome biogenesis. Early endosomes form by inward budding of the plasma membrane, which carries specific cytoplasmic cargo. Early endosomes mature into late endosomes by accumulating ILVs in their lumen and develop into MVBs.[18,19,22] MVB destinations depend on their designation molecules; they are either degraded by fusion with lysosomes or migrated toward and fused with the plasma membrane, subsequently to release into the extracellular space their ILVs, which are referred to as exosomes *(Figure 1-1)*.[11,19,27]

In most cells, MVBs carry acidic compartments that contain lysosomal hydrolases, for degradation of their content. However, some MVBs carry other molecules such as tetraspanin CD63, and lysosomal-associated membrane proteins LAMP1 and LAMP2 which are required to fuse with the plasma membrane and release the vesicle contents into the extracellular milieu.[19] Overall, it has been observed that cells host different subpopulations of MVBs. For instance, most of the cholesterol-containing MVBs tend

to fuse with the plasma membrane in an exocytic manner, approving exosomes are enriched in cholesterol.[19,20]

Multiple mechanisms can mediate ILV/MVB biogenesis. These include endosomal-sorting complex required for transport (ESCRT) components, lipids, and tetraspanins *(Figure 1-1)*. It is not known whether different processes are specific to different types of MVB or not.[19] ESCRT machinery plays an important role in the formation of ILVs and MVBs. ESCRT is composed of approximately thirty proteins that assemble into four complexes (0, -I, -II and –III) and the profoundly conserved associated proteins AAA ATPase Vps4 (vacuolar protein sorting-associated protein 4) complex and Alix.[20] ESCRT-0, ESCRT-I, and ESCRT-II recognise early endosomes with an abundance of phosphatidylinositol 3 phosphates in their membrane, ubiquitinylated cargo, and the curved membrane topology and drive membrane bud formation.[22] To complete the budding, ESCRT-III binds to the compound of ESCRT-I and II via interaction of other protein components: Alix, Tsg101, and CHMP4[22]. Then the associated proteins AAA-ATPase Vps4 complex dissociate and recycle the ESCRT machinery.[19,24]

ESCRT-independent is an alternative pathway for MVB biogenesis.[28] ESCRT-independent mechanisms require lipid metabolism enzymes or tetraspanins. Neutral sphingomyelinase (nSMase) hydrolyses sphingomyelin to ceramide, and ceramide can trigger budding of vesicles into MVBs. Also, phospholipase D2, which hydrolyses phosphatidylcholine into phosphatidic acid, induces a negative membrane curvature.[23,29] Additionally, there is some evidence to suggest that cells concomitantly depleted of the four sub-units of the ESCRT complex are still generating CD63-positive MVBs. Therefore tetraspanins with a high concentration in exosome membranes have been proposed to play role in exosome formation.[20] Tetraspanins consist of four transmembrane domains, which are connected via variable sequence defined specific protein-protein interactions. Two main tetraspanins that may play roles in exosome formation are CD9 and CD63.[22]

Several pathways are implicated in exosome formation so there is no specific surface marker related to exosomes. However, available data suggest that Tsg101, Alix, CD63, and CD9 are useful identifiers of exosomes.[18,22]

Molecular composition of exosomes

The molecular composition of exosomes is not entirely similar to their parental cell.[12,30] The most recent exosome content database (Exocarta, http://www.exocarta.org) lists 4563 proteins, 194 lipids, 1639 mRNA and 764 miRNAs that have been identified in exosomes.[12] The enrichment of specific proteins, lipids, and nucleic acids and the absence of the rest, indicate a controlling mechanism which sorts molecules in exosomes *(Figure 1-2)*.[30]

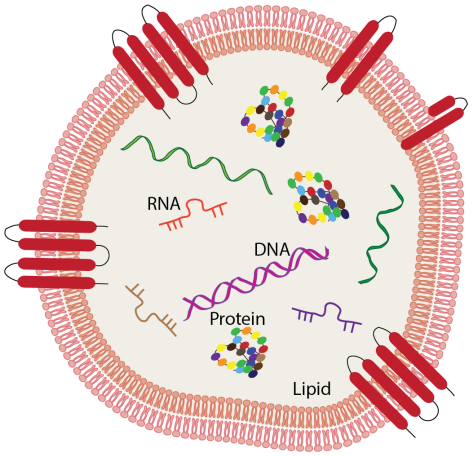


Figure 1-2 Molecular composition of exosomes

Exosomes are protective membrane vesicles with a selective package of proteins, lipids, and nucleic acids.

Proteins

The protein content of exosomes depends on the secreting cell, for instance exosomes released from antigen-presenting cells are abundant with an antigen presenting molecule, and tumour-derived exosomes contain tumour antigens.[12] Yet exosomes are also enriched for certain molecules, such as cytoplasmic enzymes (e.g., lactate dehydrogenase and peroxidases), targeting and fusion proteins (e.g., tetraspanin, lactadherin, and intergrins), chaperones (e.g., heat shock proteins Hsp60, Hsp70, Hsp90, and the small HSPs), vesicle trafficking proteins (e.g., Rab proteins, ARF GTPase, and annexins), multivesicular body biogenesis proteins (e.g., Alix, TSG101 and clathrin), cytoskeletal proteins (e.g., actin and tubulin), and signal transduction proteins (e.g., protein kinases and heterotrimeric G proteins).[14,30-32] Interestingly, proteins from intracellular organelles such as a nucleus, endoplasmic reticulum, golgi complex, and mitochondria are less abundant in exosomes.[29,33]

Lipids

Johnstone *et al.* 1989, were the first to investigate the lipid content of exosomes released from reticulocytes, yet so far the number of studies in this area is not sufficient[16]. Several studies have reported the enrichment of ceramide and its derivatives, sphingolipid, phosphatidylserine, and cholesterol, and depletion phosphatidylcholine in exosome membranes[19,34-36] independent of the parental cell. Interestingly, bismonoacyl glycerophosphate also called lysobisphsphatic acid, a membrane lipid of ILVs, is not detected in exosome membranes.[19] It is reported that sphingomyelin, a member of the sphingolipid family, plays a major role in the structure of exosomes.[18]

Enrichment of cholesterol and sphingomyelin in exosome membranes provides tighter lipid packaging and results in structural rigidity of EVs with detergent-resistant potential.[33] However, it should be noted that this enrichment is not reported in all studies; for instance, exosomes released from dendritic cells are not enriched for cholesterol. Dendritic cells have high expression of cholesterol already, and further load in their released exosomes is not expected.[34] Overall it is revealed that lipid composition in exosomes differs from their parental cells, which indicates a specific mechanism sorting specific lipid species into the vesicles.

Nucleic Acids

Exosomes carry nucleic acids, including DNA and RNA species, and preserve them from degradation.[37]

DNA

Guescini *et al.* in 2010 stated that purified exosomes from astrocytes and glioblastoma cells contain mitochondrial DNA and not genomic DNA.[38] The authors confirmed the reported mitochondrial DNA was enclosed inside the exosomes by treating purified exosomes, before DNA extraction, with DNase.[38] Further study on exosomes released by glioblastoma multiforme (GBM) cells (in both culture and tumours) revealed the presence of single-strand genomic and transposable elements.[39] Studies on exosomes released by pancreatic cancer cell lines and isolated from patient's serum revealed large fragments of double-strand genomic DNA.[40] While encapsulated DNA in exosomes has been indicated in few studies,[38,39,40] in comparison with exosomal RNA, they are not entirely characterised[40,41].

RNA

Valadi *et al.* in 2007 for the first time reported the presence of RNA in exosomes released from mast cell lines (mouse and human) and primary bone marrow-derived mouse mast cells.[42] Purified exosomes were stable in RNase and trypsin treatment, and could be internalised into other recipient cells and remain functional.[42,43] This study was the first proof-of-concept for gene-based delivery in mammalian cells.[29]

Further studies revealed that in comparison with the secreting cell, exosomes, besides carrying some parental RNA, possess a distinct RNA profile; enriched for small RNAs including significant amounts of miRNA[44,45] and depleted for ribosomal RNA[44,46]. These observations provide additional proof for the selective packaging of specific RNA species in exosomes.[44,45] Thanks to advanced technologies such as next-generation sequencing, RNA species in mammalian cell-derived exosomes are well characterized.[23] In general, exosomes are able to carry all RNA species of the cell; messenger RNA (mRNA), long non-coding RNA, small non-coding RNA (sncRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA, small nucleolar RNA, small cytoplasmic RNA, Y RNA (a type of structural RNA in ribonucleoprotein particles) and vault RNA, with a relative difference in their abundance.[29,44,45,47] Different studies show several distributions of RNA classes within exosomes. The diversity can be attributed to differences in the source of the exosomes, or reflect variations in sample preparation, exosome and RNA purification methods, RNA study techniques or even sequencing platform.[23,45]

Function of exosomes

For a long time, it was assumed that genetic information could be conveyed through two mechanisms: either vertical gene exchange from parents to the next generation or horizontal through bacteriophages and viruses.[48] During the last two decades scientists in both basic and applied research have become interested in the topic of intercellular communication, which can be broadly classified based on the distance between the communicating cells. While short-distance intercellular communication systems include gap junctions, ligand-receptor interactions and extracellular molecules such as neurotransmitters and hormones,[49,50] long-distance intercellular communication systems are achieved via particular membrane-based structures such as tunneling nanotubes, apoptotic bodies, nucleic acid binding proteins/lipids, and small vesicles.[49-51]

EVs open up a new avenue for vertical transfer of genetic information. Extracellular vesicles (including exosomes) provide a protective structure for their contents, and form a communication network between different cell types and across species.[48,52,53] Several studies have reported that exosomes are essential to maintaining the normal physiology of the body.[12] Due to the wide range of molecules in exosomes' cargo they could regulate several important biological functions such as cell growth, differentiation, immune protection and response.[51,53] Exosomes have also been implicated in the spread of diseas[54] and many other roles in pathological states.[20,48,53]

The role of exosomes in diseases

Study of the role of exosomes in **cancer** has demonstrated the impact of tumour-derived exosomes on tumour initiation, growth, progression, metastasis and the stimulation/suppression of immune responses.[12,20,42] Exosomes are also released from the **immune system**, and have immune modulatory function, with both immunosuppressive and immunoactivation effect on different steps of the immune response in their recipient cells.[51] For instance, dendritic cell, an antigen-presenting cell in the immune system, releases exosomes under different maturation states. Mature dendritic cell produce exosomes with immunoactivation ability to eliminate viruses and bacteria or eradicate tumours, while immature dendritic cell derived exosomes harbor anti-inflammatory characteristics and reduce adaptive immune activation, resulting in the promotion of tolerogenic immune responses in transplantation and autoimmune disesases.[55]

Several reports have demonstrated that different cell types of the **nervous system** release exosomes with neuronal communication roles in the normal physiology of the nervous system as well as neurodegenerative disease.[51] For instance, the presence of mature neuron-derived exosomes has been reported in the vicinity of synaptic connections with a normal physiological function.[56] Conversely, neuron shed exosomes may play a detrimental role by spreading pathogenic agents or degenerative proteins like beta-amyloid in Alzheimer's disease.[56,57] Beside healthy brain cells, exosomes released from glioma can also transfer oncogenic proteins among cancer cells.[57] Therefore, exosomes released within the CNS can modulate signaling pathways, and spread neurotoxic proteins.

Roles of exosomes in therapeutics and diagnostics

Exosomes carry a particular package of cargo which reflects their cellular origin and plays roles in both physiologic and pathologic conditions. Moreover, these membrane-bound vesicles are small and flexible and can cross biological barriers such as the BBB and target specific organs/tissues. These characteristics plus recent technological advancement make exosomes a distinct diagnostics and therapeutic candidate,[12,51] so several studies have attempted to exploit exosomes' potential.

Exosomes for therapeutic approaches are mainly considered as a new **delivery system** that can be loaded with several molecules (e.g., nanoparticles, oligos, and drugs), and modulate the function of target cells with low toxicity, high stability, and intrinsic homing capacity.[12] Exosomes capability regarding transport of endogenous mRNA and miRNA to other cells resulting in recipient-cell protein product modulation had been previously described in both mouse and human cell culture studies.[41,42] Based on this knowledge, in 2010, Alvarez-Erviti *et al.* examined the possibility of delivering loaded exosomes with exogenous cargos small interfering RNA (siRNA) to a particular tissue or cell types in vivo. In this study, modified exosomes with specific targeting proteins were used to deliver siRNA to a mouse brain via a systemic injection. The results were comparable to gene knockdown with a potential therapeutic approach to Alzheimer's disease.[8] Since then, several studies have considered exosomes reliable vehicles to shuttle exogenous cargos for therapeutic purposes. However, there are some technical, functional and safety features to be solved and addressed.[51]

Another therapeutic approach is based on the presence of surface antigens on tumour-derived exosomes, which stimulate the immune system to respond against the tumours. These exosomes could be utilised to induce antitumor immunity in patients.[12,20,41] Even though experimental evidence indicates the use of **exosomes for cancer vaccine** is a safe and successful strategy, these are some limitation because exosomes must be prepared from treated patients resulting in variation in the yield of exosomes and the amount of received treatment.[51]

It has also been reported that the number of exosomes is elevated in samples from patients suffering cancer,[29,51] inflammatory[20] and neurodegenerative[54,58] conditions compared to healthy individuals. Therefore, one therapeutic strategy would be **modulating the number of secreted exosomes** through inhibiting their formation, release, or uptake by target cells.[12,20]

Since exosomes circulate in almost all biofluids, they have been considered as an easily accessible **diagnostic biomarker** to indicate pathological conditions. Several studies have investigated the level of circulating exosomes as well as their cargo (e.g., miRNA, long non-coding RNA and proteins) as potential biomarkers.[12]

Exosome purification

Exosomes have received great attention in recent years due to their vital roles in cell-to-cell communication.[59] Therefore, the isolation and quantification of exosomes has become indispensable in both basic research and clinical applications.[59] Extracellular vesicles including exosomes can be isolated from cell culture-conditional media or body fluids including plasma, serum, saliva, urine, milk and cerebrospinal fluid (CSF).[26,47] Given that exosomes can cross the BBB, it is thus likely that at least some of the circulating exosomes in a patient's blood sample are derived from affected CNS cells or the associated inflammatory milieu.[8,9] Therefore, the presence of exosomes and their cargo, such as miRNAs in body fluid has incited considerable interest in their use as biomarkers for many diseases including MS and brain cancer.[41,60]

The comparison between plasma and serum as a source of exosomes demonstrated diversity in the final results, which is related to either the clotting process of serum (exosomes trapped within the clot) or the existence of heparin in plasma (exosomes-heparin complexes).[61] Although plasma had initially been considered the best source for exosome purification compared to serum[61], further studies have demonstrated higher yields for extracted miRNAs from serum exosomes in comparison with plasma[29,62].

Several techniques have been employed for the isolation of various EV subpopulations utilising their particular features, such as density, shape, size, and surface proteins.[59,63] The techniques mainly used for exosome isolation include ultracentrifugation, precipitation, immunoaffinity capture-based technique and size exclusion chromatography.

Ultracentrifugation

Johnstone *et al.* in 1987, developed the first protocol to purify reticulocyte exosomes from tissue–conditioned medium, which was based on differential centrifugation.[19] Centrifugation applies a force to separate particles in a suspension according to their physical properties and the density and viscosity of the solvent. Ultracentrifugation

(UC) is a centrifugation process with exceptionally high centrifugal forces to fractionate small bioparticles.[59] Ultracentrifugation-based exosome isolation is the most commonly used and reported technique and considered as the gold standard protocol.[59,63] To isolate exosomes by differential ultracentrifugation, a series of centrifugation cycles of different forces and durations is applied. First, bigger particles and debris are eliminated by low-speed centrifugation for a short time-period. Then, the smallest vesicles are sedimented by higher-speed ultracentrifugation with extended time.[19,59,63]

Due to the heterogeneity of exosomes, the final result of ultracentrifugation is not the enrichment of pure exosomes, and it is contaminated with other small EVs and protein aggregation.[19] Overall, this method suffers from low recovery (5–25%) and purity for exosome purification. To date, this protocol has been optimised in many ways such as adding an extra density gradient centrifugation step, combining ultrafiltration membranes or size exclusion chromatography to exclude soluble proteins.[59,63] One way to optimise ultracentrifugation-based exosome purification is ultrafiltration, using hydrophilic polyvinylidene difluoride membranes of different pore sizes, with the first and second low-speed centrifugation steps.[63] Ultrafiltration is an independent size-based exosome isolation technique, which separate particles based on their size and weight.[59] Substituting ultrafiltration instead of centrifugation reduce the risk of bigger particles fragmenting into smaller vesicles.[15] Alternatively, in density gradient ultracentrifugation, exosomes are isolated based on their size, mass and density in a pre-constructed density gradient medium of sucrose or iodixanol (Optiprep™) in a centrifuge tube. This method allows exosomes to float during centrifugation through the density gradient medium and move as an individual zone (density region 1.10 and 1.21 g/ml), which can later be recovered by fraction collection.[59,63,64] While adding a density gradient centrifugation step improves the total yield of purified exosomes, it is often contaminated with viruses or large microvesicles with comparable sedimentation velocities.[47]

In conclusion, sequential ultracentrifugation alone is not sufficient to separate EV's based on their size and further combinations of alternative methods, gradient centrifugation or ultrafiltration, may help to overcome the limitations.

Exosome precipitation

This method relies on changing exosomes' solubility or dispersibility using a commercial agglutinating agent, such as polyethylene glycol to sediment exosomes.[59] Samples are mixed and incubated with polyethylene glycol-containing reagents followed by either low-speed centrifugation or filtration to isolate the precipitate containing exosomes.[63] Currently, several biotechnology companies have developed compatible exosome precipitation kits for a variety of samples including culture medium, serum, plasma, and urine.[59,63] Although this method is easy to use without any specific technology requirement, it co-precipitates other EV populations, proteins, and polymeric materials in the isolated samples. These can interfere with subsequent experiments.[65]

Immunoaffinity capture-based technique

The presence of proteins in the membrane of exosomes can be employed to isolate exosomes by immunoaffinity interactions between these proteins and their antibodies.[59] Therefore, immunoaffinity capture-based techniques utilise the monoclonal antibody coated on magnetic beads directed against exosome-specific antigens to purify certain exosome sub-populations.[47] This method can be used as an individual experiment to colocalise exosomes or as a complementary technique to further purify and select a particular sub-population of exosomes.[63] Although this method prepares high-quality exosomes, it is limited to isolating only a subpopulation of marker-positive exosomes, while excluding the negative exosome population (for the examined membrane protein) and the yield might be contaminated with other positive extracellular vesicles.[66,67]

Size-exclusive chromatography

Another size-based separation technique to isolate exosomes is single-step size exclusive chromatography (SEC) or 'gel filtration'.[68] A SEC column is a syringe with 10 ml sepharose resin CL-2B, which is equilibrated with Phosphate buffered saline-citrate 0.32% (w/v).[68] This column has a porous stationary phase which is utilised to sort macromolecules and particles based on their size. Small components in a sample are able to pass through the pores resulting in late elution, while components with large hydrodynamic radii such as exosomes, are excluded from entering the pores and are eluted in earlier fractions. Therefore SEC can be used to isolate exosomes from sera or plasma by fractionating vesicles based on their size. In general, the early fractions contain vesicles around 70 nm in size and delayed ones are mostly other

contaminations. Accordingly, the use of SEC results in excluding proteins and high-density lipoproteins.[69] The combination of SEC with other techniques such as ultracentrifugation is also applicable to reduce the initial volume of samples and improve the final yields of exosomes.[59]

In conclusion, various conventional strategies are available to purify exosomes and there is currently no consensus on a gold standard method. Despite the overall similarities, the isolation protocols for each method must be validated based on the prime aim of the research, the sample type and the downstream applications.[26,63]

Exosome heterogeneity

It has been shown that the molecular composition of exosomes is not only cell-type dependent but can also depend on the activation status of the parental cell. The heterogeneity in exosomes is reflected in their lipid, protein and also nucleic acid compositions. Despite these findings, the current limitations in exosomes isolation techniques mean that the majority of studies employ the bulk isolation of exosomes.[70] For example, using ultracentrifugation for isolating exosomes separates vesicles according to their size and density.[59] Even more specific methods such as the immunocapturing technique relies on purifying a subpopulation of exosomes based on a specific protein displayed on their surface, (e.g., CD63-positive), is equally problematic as it introduces bias into the subpopulation of interest and neglects the fact that there are diverse subpopulations exosomes.[63] There are new techniques, which allow the detection and isolation of exosomal subtypes and rely on known and common exosomal or disease specific markers and can be used for applications such as therapeutic drug delivery vehicles and for cancer vaccination. However for the de novo identification of any unknown exosomal subtypes, such as for biomarker studies, exosome purification remains challenging.[71]

Exosome characterisation

Regardless of the employed method, the isolated vesicles should be characterised to demonstrate the presence of vesicles and assess their purity.[60]

Size and morphology

In general, the isolated vesicles should be visualised to characterise individual EVs and indicate their heterogeneity.[26] The size of EVs are too small to fit within the resolution

threshold of optical microscopy[22,19,72] and classical flow cytometry[15,34,73]. Classical flow cytometry does not distinguish vesicles >200 nm from noise, so that electron microscopy and light scattering techniques are adopted to observe and confirm the size and morphology of exosomes.[19,59]

Transmission electron microscopy

Transmission electron microscopy (TEM) studies demonstrated a cup-shaped appearance as the natural morphology of exosomes with a diameter of 50–100 nm over the past 10 years.[19] However, cryo-EM show exosomes have a round-shape morphology, and a cup-shaped feature is actually an artifact that forms during preparation of the exosomal samples for TEM. In TEM studies, heavy metals such as uranyl acetate and osmium tetroxide are utilised to increase the contrast of EV samples, which results in dehydration of samples and induces shriveling of cellular structure.[15,19,74] Despite this drawback, TEM is the standard method in EV studies to determine size and structure of single vesicles and to represent particles heterogeneity with wide field images encompassing multiple vesicles.[15,19,26]

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a light scattering technique, which provides the size distribution and concentration of nanoparticles based on their Brownian motion and size.[19,72,75] NTA tracks the random movement of particles illuminated by a laser beam and uses light scattering to calculate their diameter.[19,75] The movement rate of particles is related to their size (smaller particles move faster), the viscosity and the temperature of the solvent.[59,75] NTA is a fast and simple method to analyse scores of particles and since 2011 was accepted as one of the standard methods for EV characterisation.[74] Nevertheless, it does not provide a precise result for vesicles since it counts all particles with similar size including membrane vesicles and co-isolated artifacts such as protein aggregates.[19,26] Therefore, a combination of both TEM and NTA results are required to characterise EVs size, morphology, and concentration.[26]

Membrane protein composition

Alongside specific proteins from the parental cell,[14] several studies reported a defined set of cellular proteins in EVs, regardless of their parent cell type[23,73]. Surface membrane proteins enriched in mammalian cell-derived exosomes include adhesion molecules such as integrin and tetraspanins (CD9, CD63, CD81) which regulate cargo-loading process, antigen-presenting proteins, major histocompatibility complex (MHC)

class I and II, and membrane transport/fusion proteins (annexins and flotillins).[30,31] These observations affirm the specificity of exosomes' endosomal formation.[14] Since another type of EV might represent the same proteins,[29] these proteins are considered as exosome-enriched proteins rather than exosome-specific markers.[26] Therefore, it is suggested to determine at least three enriched endosomal origin proteins with a semi-quantitative method in exosome preparation.[26] Western blotting is antibody-based detection technique to identify a particular protein, while proteomic analysis with mass spectrometry covers a broader approach.[76] Mass spectrometry has improved in the last 20 years and several thousand EV proteins rom various sources have been deposited in the free web-based EVpedia (http://evpedia.info) database.[77]

Non-coding RNA

The genome of multicellular eukaryotes, unlike prokaryotes, is transcribed into a diversity of RNAs, the vast majority of which (>90%) do not encode proteins, but are non-coding RNAs (ncRNAs).[78,79] Genome sequencing projects have revealed that the number of protein-coding RNAs is relatively constant across vertebrates and does not indicate the complexity of the organism, whereas the developmental programming is associated with the proportion of non-protein coding RNAs (e.g., the human genome contains 98% non-protein coding RNAs).[80] This observation suggested that ncRNAs perform a significant function in genetic programming during differentiation and development, through regulation of gene expression.[81] The first ncRNA with gene expression regulatory function, miRNA, was identified about 25 years ago (in 1993), with the discovery of *lin-4* in the nematode worm in *Caenorhabditis Elegans*.[82,83] This finding followed by the discovery of RNA interference in 1998, highlighted the importance of ncRNAs.[82] Since then, a broad spectrum of ncRNAs with multiple functions has been discovered[84,85] and, based on the size and synthesis mechanism, categorised into two main groups, the small and long ncRNAs[78].

Small ncRNAs (>200nt) are generated by the post-transcriptional processing of longer transcripts by endogenous RNases.[86] sncRNAs have been classified into various categories by their length, biogenesis, function and structural or sequence feature.[78] Three subclasses: siRNAs, piwi-interacting RNAs, and miRNAs are mainly involved in gene silencing in the cell through RNAi interference mechanisms.[86] These sncRNAs (~20–30 nt) carry nucleotide sequence complementarity, which mediates regulatory

control after incorporation into a complex of proteins (RNA-protein complexes).[86] SiRNAs (21–24 nt) are double-strand RNA with a gene silencing function, which induces transcript degradation in a sequence-specific manner.[87,88] Endogenous siRNAs selectively target double-strand RNA viruses and transposons to protect the eukaryotic cells.[78] Both siRNAs and miRNAs function as post-transcriptional gene regulators and use the same enzymatic mechanism to be activated. Thus, synthetic siRNAs became a therapeutic tool for several diseases and disorders, yet certain drawbacks such as low stability under *in vivo* conditions remain to be addressed.[78,82,87] Piwi-interacting RNAs (28–32 nt) were discovered in 2006, and play a pivotal role in the physiological modulation of spermiogenesis.[82] Piwi-interacting RNAs protect the genome integrity of germ cells by silencing transposons and other random integrates, and are involved in gametogenesis by guiding DNA methylation to suppress the expression of repetitive elements. PiRNAs function, unlike siRNAs and miRNAs, are associated with the piwi subclass of the Argonaute superfamily, not Argonaute protein, and produce independently of Dicer.[78,89]

Long ncRNA (<200 nt) were later introduced as large portions of the mammalian noncoding transcriptome, with little or no protein coding capacity, which also regulate gene expression and relevant to evolution.[84,85]

MicroRNAs

MicroRNAs (miRNA) — from 17–24 nt — are the most extensively studied sncRNAs, and are involved in almost every biological process—both physiological and pathological.[90,18,84] MicroRNAs regulate gene expression in one-third of human genes by pairing to their complementary sequence, located in the 3′ untranslated region (UTR) or open reading frame of target mRNAs, and mediate post-transcriptional gene silencing.[83,84,91] RNA polymerase II or III transcribe miRNA genes into monocistronic (single) or polycistronic (cluster) precursor miRNA named primary miRNA (pri-miRNA) with a phosphorylated 5′ end and a short overhang on the 3′ end, and a double strand stem-loop shape. In the nucleus pri-miRNA binds to the dsRNA-binding protein—DGCR5 (DiGeorge Syndrome chromosome region)—of the Drosha complex and is digested by the complex's enzyme -RNase III enzyme Drosha- to form short (60–70 nt) hairpin precursor miRNAs (pre-miRNA). Pre-miRNAs are exported to the cytoplasm by exportin5-Ran-GTP complex and further digested by Dicer, another

member of RNase III protein, into mature ~22 nt double-stranded miRNA (guided strand/passenger strand of the same hairpin structure). One strand of mature miRNAs is incorporated into RNA-induced silencing complex as a guide strand to silence target mRNA *(Figure 1-3)*.[78,92,93]

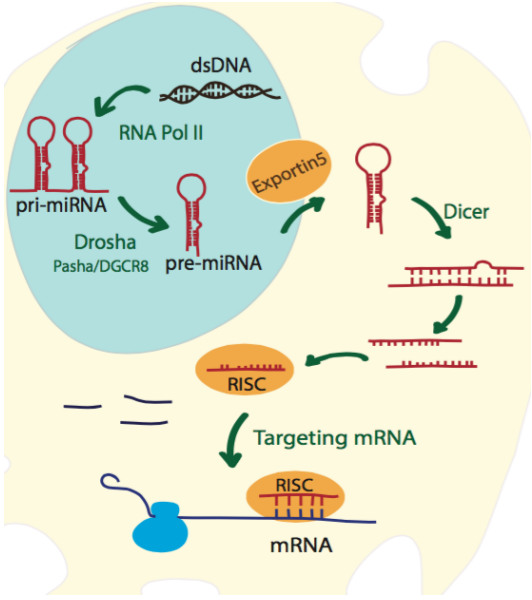


Figure 1-3 miRNA biogenesis

MiRNA genes are transcribed as Pri-miRNAs. In the nucleus, the complex Drosha processes Pri-miRNAs into Pre-miRNAs, which are transported to the cytoplasm by the Exportin5 complex. IN the cytoplasm Dicer processes the pre-miRNA to form mature double-stranded miRNA. One strand of mature miRNA is incorporated into the RNA-induced silencing complex.

miRNAs recognize their specific target mRNAs by 2–7nt in the 5′ end called the 'seed sequence'. The degree of the miRNAs and target mRNAs complementarity determines the result: either translational repression or mRNA degradation. The relatively short complementarity sequence between miRNAs and mRNAs imply a single miRNA can target many genes and mRNAs harbor multiple miRNA-binding sites in their 3′UTR.[79,84,90]

Genome location of miRNAs can be intergenic, intronic, or exonic. DNA sequences between gene-coding DNA are independent transcription units called intergenic regions that may or may not encode regulatory functions. Many miRNAs are encoded in the intergenic region with miRNA-specific promoters. Intronic miRNAs are processed from the introns of the host genes either: protein-coding genes or long non-coding RNAs. And exonic miRNAs are mostly process from noncoding genes. The host gene promoter transcribes intronic and exonic miRNAs.[90,93]

Several studies reported the contribution of ncRNAs to not only regulating almost all steps of gene expression but also the cellular processes involved in development and disease.[78,79] Due to the purpose of our study, this section is concentrated on miRNAs.

Functions of miRNAs

The role of miRNAs in disease and diagnosis

Non-invasive tools to diagnose and monitor the progress of diseases has long been a goal of research.[94] Animal examinations, particularly mouse disease models, reveal that miRNA levels change to trigger disease and also as a direct consequence of disease onset or progression and as such could be used for prognosis and potential therapy. Therefore, miRNAs are at the center of attention for studies of numerous different diseases.[84] Based on version 21 of miRBase (http://www.mirbase.org), the main reference miRNA database, over 35,800 mature miRNAs in 223 different species have been identified.[95] Each miRNA can regulate more than several hundred potential target mRNAs.[93,96] Also, several single nucleotide polymorphisms,[97] length and sequence heterogeneities provoke enormous polymorphisms in miRNAs, which might result in phenotypic diversities and are responsible for or contribute to the pathogenesis of disorders[95,98].

Unbiased high-throughput techniques (e.g., microarray and sequencing) enables the screening of all known miRNAs while eliminating the possibility of missing some affected miRNAs.[85] This achievement leads to better understanding of the association between miRNAs and numerous human pathologies. Therefore, altered miRNAs, as a result of diseases could either be detected for diagnosis or be used as therapeutic targets.[84]

MicroRNAs are a promising tissue-based biomarker for cancer research.[99] Several studies report the abnormal expression of miRNAs in cancer initiation and progression – they may act to inhibit tumour suppressor gene expression or activate oncogene expression. The study of aberrant miRNA expression profiles in the body fluids of patients with cancer represents an innovative way to identify new biomarkers for disease detection and progression.[15] Importantly, miRNAs signatures with highly tissue-specific origin could lead to efficient identification of metastatic cancers of unknown primary origin.[100,101]

In prostate cancer, several studies report the value of serum and plasma circulating miRNAs in the detection and prognosis of prostate cancer.[100] Deregulated miRNAs have been implicated in several processes in this cancer including cell proliferation, differentiation and progression. Despite variation in patients' age, ethnicity, and the different balance between local and advanced prostate cancer, the expression of miR-141, miR-375 and miR-21 were reported significantly higher in patients than in matched healthy individuals, repeatedly.[100,102]

In 2011, Kanemaru et al. for the first time demonstrated a diagnostic role of circulating miRNA expression in melanoma by reporting an increase in the serum level of miR-221 in patients with metastatic disease. In later studies, a serum-based signature of four miRNAs (miR-15b, miR-30d, miR-150 and miR- 425) were found to predict recurrence of cancer; co-detection of miR-185 and miR-1246 was found to distinguish patients with metastatic melanoma from healthy individuals.[103]

MicroRNAs also perform a critical function to modulate both the innate and acquired immune systems and develop autoimmune reactions.[104] It has been reported that Dicer enzyme with regulatory responsibility in RNA biogenesis is required for T-cell function and removing Dicer enzyme results in autoimmune disorders.[104] Moreover, several specific miRNAs are present within the immune system and have a dramatic impact on autoimmune responses through their regulation of inflammatory T cells.[105] For instance, miR-155 promotes inflammatory responses, while miR-146a, in contrast, limits T-cell activation and mediates immune suppression.[105,106] Therefore, these miRNAs can be considered as therapeutic or diagnostic tools. It has been suggested modulating these miRNA's expression level with specific and targeted approaches might be a therapeutic means to treat autoimmune disease.[106]

Development of the CNS and generating interconnection between distinct cells within the CNS require coordinated events (e.g., transcriptional networks and cell signaling) and multilayered regulatory networks (e.g., electrochemical signals).[93] In this regard, abundant numbers of miRNAs have been revealed in the brain, compared to other organs, contributing to neuronal development regulation.[107] These miRNAs are highly tissue-specific, and even distinct cell types in the CNS shed miRNAs with specific patterns and function.[108] The first reports translating profiles of deregulated miRNA studies to the brain identified some brain enriched miRNAs such as miR-9, miR-29a, miR-125, miR-128, miR-134, and miR-137. A number of these deregulated miRNA

(miR-9, miR20a, and miR-132) have been reported to correlate with Alzheimer disease.[109]

The resident innate immune cell of the CNS, microglia, performs as the primary defender in immune surveillance, which in response to brain injury or other stressors activates inflammation. Dysfunction in microglia results in nonresponsive pro-inflammatory activity leading to neurodegeneration.[108,110] Microglia are enriched for specific miRNAs (e.g., miR-124 and miR-155) with heterogeneous expression in different brain regions. For instance, miR-155 promotes microglia pro-inflammatory function while miR124 inactivates microglia and reduces CNS inflammation. These findings indicate miRNAs regulatory roles in the behavior of microglia, and their effect on inflammation and related neurological diseases.[110] Therefore, modulating miRNAs to skew the behavior of microglia based on each disease's specific requirement is a novel therapeutic strategy.[84]

Although several studies have confirmed that miRNAs have a biomarker potential, free circulating miRNAs are fluctuating in their stability.[111] Conversely, miRNAs related to extracellular vesicles are a better candidate to study as they carry a selective package of miRNAs, and reflect their cell or tissue origin.[111] Thus, in this study, we have focused on exosomal miRNA as a valuable biomarker to monitor the neurological diseases and assess their treatment, which can be measured in the serum in a minimally invasive manner.

Multiple sclerosis

Background

Multiple sclerosis (MS) is an idiopathic inflammatory, demyelinating, and progressive neurodegenerative disease of the CNS.[112] It is a chronic disease with a widely variable clinical course that results in functional and cognitive deterioration over time, and a reduced lifespan. MS is the most common neurological disease of young adults, and disproportionally affects women. This chronic disease results in ongoing functional deterioration and increasing disability.[113] MS affects 2.5 million people worldwide[114] and over 23,000 people were living with MS in Australia in 2013[115].

The reported incidence of MS has increased in the last five decades, especially in women. This is partially attributable to new diagnostic technologies and increased awareness. Alternatively, environmental factors (e.g., smoking) and reproductive factors (e.g., pregnancy) may contribute to the female to male ratio increase.[116,117]

MS was first characterised as a distinct disease in 1868 by Jean-Martin Charcot, who correlated the clinical features of MS with pathological changes noted post-mortem.[118] Despite being described almost 150 years ago the cause of MS still remains unknown. The disease primarily affects the myelin sheath around central axons, slowing axonal conduction and leading to a variety of deficits based primarily on the location of the demyelinating lesion(s). It has become increasingly evident that not only the myelin sheath is affected in MS; there is significant and cumulative axonal damage and even neuronal cell loss in chronic MS, and these latter effects correlate most closely with long-term motor and cognitive disability. The immune system is clearly dysregulated in multiple sclerosis; however, it is unknown whether MS represents a primary autoimmune disease targeting the CNS, or whether there is a primary abnormality within the CNS that recruits a secondary adaptive immune response (the 'inside-out' hypothesis).[112,119]

With modern treatments, MS rarely develops into a life threating disease: 95% of patients have a normal life expectancy. However, disease symptoms significantly affect the patient's quality of life: 43% of MS patients suffer from progressive motor deterioration that leads to physical and mental disability. This not only imposes increased dependency on affected individuals but also a heavy financial burden on their families and society.[115]

MS development and propagation

MS is a chronic inflammatory autoimmune demyelinating disease of the CNS, which involves the brain, spinal cord, and optical nerves and causes motor, sensory, and cognitive deficits. Initially, remyelination—a spontaneous repair in the adult CNS—recovers the demyelination. However, the pathology of the disease changes over time and neurological dysfunction becomes dominant.[120,121]

MS pathogenesis is unknown and the current theory is that it begins with an inappropriate cascade of activated T cells into the CNS by crossing the broken BBB.

Activated T cells produce inflammatory cytokines and trigger an immune response against myelin, oligodendrocytes (myelin-forming cells), and surrounding tissue, resulting in the formation of demyelination plaques and axonal degeneration. The demyelinated axons lead to weak conduction velocity of action potentials and in extreme cases, may completely inhibit the signaling pathways.[122] Demyelination and axonal degeneration patterns are shown in *Figure 1-4*.

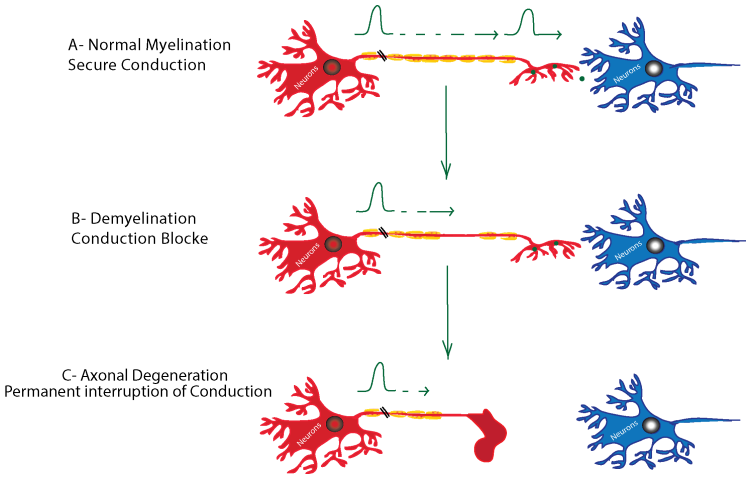


Figure 1-4 Demyelination pattern

A) The axon of a healthy nerve cell covered with myelin sheaths that contribute to conducting signals from the nervous system to the rest of the body. B) Disruptions in myelin sheaths result in slowing down or halting signal conduction. C) In the case of axonal degeneration, demyelination is irreversible, and signal conduction is lost.

MS plaques (MS lesions) can occupy different spots in the white matter, including the spinal cord.[120] Magnetic resonance imaging[123] is used to diagnose these lesions in MS suspected patients. There is a growing recognition that grey matter demyelination is also involved in disease propagation. While the lesions in the grey matter are mostly undetectable with traditional magnetic resonance imaging (MRI), the results of immunohistochemical studies in the past decade have shown intense involvement of the grey matter of the CNS in the pathology of MS.[124] Thus, MS is a complex disease with a wide range of symptoms affecting several parts of the body with varying severity.[125]

Etiology of MS

MS is thought to have a multifactorial etiology. To date, no causative agent has been identified in MS; susceptible genes, an abnormal immune system, and specific

environmental exposure have all been proposed as factors involved in the causation, exacerbation, and prognosis of MS (*Figure 1-5*).[126]

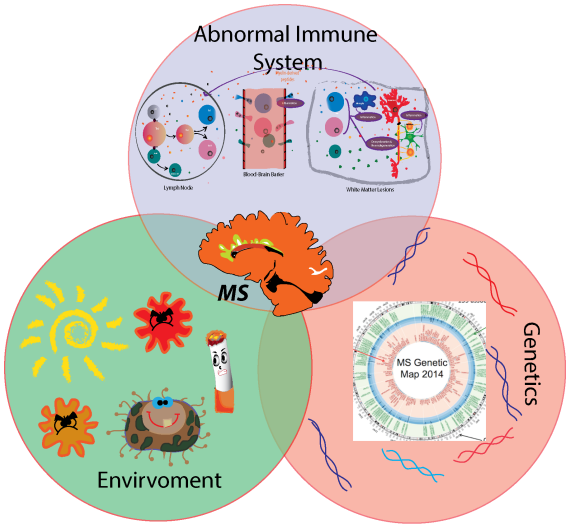


Figure 1-5 Etiology of MS

Multiple sclerosis is a complex neurological condition with an unknown etiology. A variety of factors are involved in developing MS; abnormal immune system, environmental components and susceptible genes, and interactions between.

Twenty five per cent of MS patients have a family history of the disease in a first-degree relative. However, no single causative gene has been identified. This suggests that MS is not a classic Mendelian heritable disease. Instead, it is more likely to be polygenic, and/or a combination genetic predisposition and environmental stressors. Interestingly, epidemiological data also has shown that imprinting or parent-of-origin effect in MS plays a substantial role.[127]

The first evidence of the association between human leukocyte antigen (HLA)[128] haplotype and MS susceptibility was shown in 1972[129]. Later this association was referred to as HLA-DRB1*1501, a haplotype with significant genetic effect in MS (heterozygosity odds ratio (OD) of 2.7 and homozygosity OD 6.7). Despite the strong phenotypic effect of HLA-DRB1*1501 on the disease, this association is not straightforward. In fact, a combination of different haplotypes has a positive or negative impact on MS. For instance, HLA-DRB1*08 in combination with HLA-DRB1*15 doubles the risk of MS. Conversely, HLA-DRB1*14, as a protective haplotype, completely nullifies the increased risk of HLA-DRB1*15.[130] It has been showed that

between 20–60% of the genetic susceptibility in MS is related to the HLA locus. In addition to the HLA regions, two other risk loci (IL-7RA, interleukin 7 receptor alpha, and IL-2RA) have been identified in promoting lymphocyte growth and differentiation. More than a hundred (102) single nucleotide polymorphisms have also been shown to affect MS disease risk, mostly through affecting the immune function.[131]

Many studies demonstrate that age and gender play a definitive role in MS susceptibility. MS occurs most commonly in young people, between ages 20 and 40 (onset before 10 and after 55 is rare), and in women.[122,132] Over the past century, the female predominance of MS has increased significantly. The mechanisms underlying the observed gender specificity are still largely unknown. Factors such as the role of hormones, differences in the male and female immune system and the CNS, genetic and epigenetic factors may be relevant.[126]

MS susceptibility is also strongly influenced by the environment. For example, geographical locations are closely associated with MS risks. Distance from the equator and the climate correlate with the prevalence of MS, with the lowest rate of incidence in Africa, South America, and Asia, and the highest incidence in Scotland and parts of Scandinavia and Canada.[127] This fluctuation may be partially explained by common genetic factors of the ethnic groups who live in these risk areas. However, migration has been shown to change the likelihood of developing MS especially for migrants who have relocated at a young age.[131] Similarly, exposure to sunlight has been shown to decrease MS susceptibility. It is thought to have a protective effect mediated by ultraviolet radiation, as the primary source of vitamin D for most people. Vitamin D is important for both normal physiology and several autoimmune diseases.[133]

Systemic infection during childhood or early adulthood is thought to be another etiological factor for MS. This is because increased level of IgG and the presence of alkaline oligoclonal bands in the cerebrospinal fluid (CSF) are detectable in almost all of the MS patients (95%). Direct viral damage to brain cells or the productions of antibodies, which attach the myelin, are hypothesized to act as a trigger for demyelination in MS patients. Human Herpes Virus-6 (also known Epstein Barr Virus; EBV) is the best known candidate for MS causation.[134] Current or past cigarette smoking also increases MS risk. While there is no known mechanism for this, smoking has a direct effect on the immune system.[133]

Overall, the exact causes of MS are unknown, although solving the complex genetics underlying the disease as well as the environmental risk factors such as EBV infection, smoking and vitamin D status may help to achieve a better understanding of MS etiology.[126]

Immunopathogenesis of MS

MS is a common debilitating disease characterised by inflammation in CNS. It is associated with focal plaques of primary demyelination and distributed neurodegeneration in the brain and spinal cord.[126] There are many conflicting theories to explain MS. The most widely accepted theory is that multiple sclerosis begins with an inflammatory cascade in the CNS. Autoreactive T- and B- cells from the peripheral immune system infiltrate the CNS by crossing the BBB and trigger an immune response against myelin, myelin-forming cells (oligodendrocytes), and the surrounding tissue (the outside-in hypothesis) *(Figure 1-6A)*. Alternatively, there is a hypothesis that a primary infection or neuronal disruption within the brain can act as an initial trigger, and inflammation occurs as a secondary response followed by tissue damage and disease (the inside-out hypothesis) *(Figure 1-6B)*.

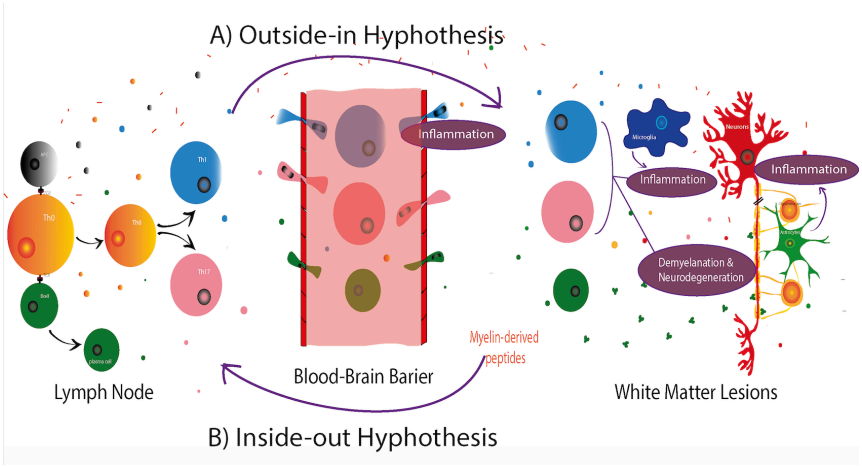


Figure 1- 6 MS: the demyelination disease with an immune system disorder and an unknown trigger

A) Outside-in hypothesis: MS is an autoimmune disease targeting the CNS. B) Inside-out hypothesis: There is an abnormality in the CNS that initiates the disease and an activated immune system response to it.

However, there is no known autoimmune reaction or infectious agent specific to MS that can provide evidence for either hypothesis of MS initiation. Therefore, it is still

largely unknown whether MS represents a primary autoimmune disease targeting the CNS, or if there is a primary abnormality within the CNS that recruits a secondary adaptive immune response.[135]

Types of MS

Despite the prevailing dogma in neurology that the CNS was relatively stable in structure, evidence from the 1990s demonstrated that CNS is capable of plasticity and repair. Indeed, remyelination of CNS axons is now a well-documented phenomenon.[121] This phenomenon is best seen in the white matter of the brain and spinal cord in MS patients. Remyelination occurs in two major phases: oligodendrocyte progenitor cells colonise in the lesions and then differentiate into myelination oligodendrocytes. The process of remyelination cannot keep pace with deterioration, so inadequate remyelination is generally observed in the MS brain and spinal cord. In this regard, the balance between number of oligodendrocytes and macrophages in lesions play a vital role.[121]

Patients with MS present wide range of clinical manifestations. The type and severity of symptoms depend on the location of scar tissue and the extent of demyelination. Different patients demonstrate a variety of symptoms and even the same patient shows highly variable duration and expression of symptoms over time.[136] Based on the course of the disease MS patients are categorised into four major groups. Clinically isolated syndrome is the first neurological presentation of the disease with inflammatory demyelination characteristics that can be optic neuritis, incomplete myelitis, or brainstem syndrome *(Figure 1-7A)*.[137]

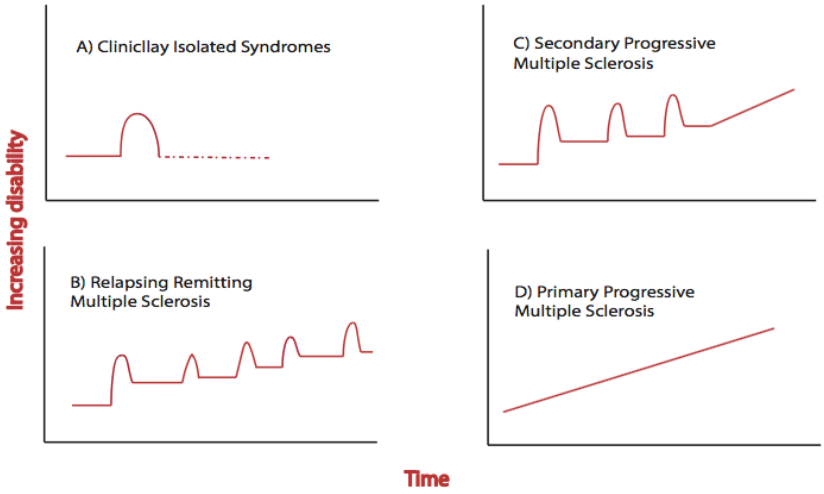


Figure 1-7 Different types of MS

A) Clinically isolated syndrome is the first episode of neurological disorder and demyelination with a chance of developing into MS. B) Relapsing remitting multiple sclerosis (RRMS) the most common phenotype of MS with various numbers of disease symptom flare ups (relapses) and spontaneous recoveries (remitting). C) Secondary progressive multiple sclerosis (SPMS) in which symptoms of the disease progress constantly. D) Primary progressive multiple sclerosis (PPMS) without relapsing remitting stage and disease diagnose in a progressive stage.

People presenting these symptoms may or may not meet MS diagnosis criteria and develop the disease.[137] The disease in 85% of patients starts with a relapsing- remitting course.[138] The symptoms of disease flare-up (relapse) and then in a period of days or weeks the symptoms recover to very low levels- symptoms may improve or disappear *(Figure 1-7B)*. After 10 to 15 years the disease in the majority of RRMS patients gradually become progressive with or without acute exacerbations during the progressive course, named SPMS *(Figure 1-7C)*.[139] This transformation occurs with no clear clinical, imaging, immunologic or pathologic criteria. Another spectrum of progressive MS is primary progressive MS (PPMS) seen in 10-15% patients. These patients miss the relapsing- remitting stage as a clinical manifestation, so symptoms tend to worsen consistently from the disease onset *(Figure 1-7D)*.[140] The absence of exacerbations prior to clinical progression separates secondary progressive MS (SPMS) from PPMS[141], while both are considered as progressive MS with similar clinical and imaging features[142,143].

Disease management

A variety of pharmacological therapies are available for MS: symptomatic treatment and disease modifying therapies (DMT).[144] While symptomatic therapeutics focus on relieving specific symptoms such as fatigue, DMTs suppress the peripheral adaptive immune response to reduce the inflammation and alter the course of the disease by minimizing the frequency of relapses, the number of MRI lesions and slowing down the progression step.[145] The first DMT medication, interferon beta-1b, was introduced in 1993 and since then many other treatments have become available, which can be categorised as platform (self-injectable) medications (e.g., Plegridy, Rebif, and Copaxone), infusions (e.g., Natalizumab and Alemtuzumab), and oral medications (e.g., Fingolimod and Tecfidera).[146] DMTs have been reported to have greater efficacy in RRMS patients, mainly in early initiation compared to delayed commencement, rather than in MS patients in progressive stage.[147]

DMTs can also be divided into two groups based on their mechanism of action: low-efficacy, and high-efficacy. While high-efficacy treatments are more efficient in reducing relapses and inverting brain atrophy, the common strategy to manage MS begins with low-efficacy DMTs with fewer side effects. The therapy intensifies to high-efficacy DMTs in patients demonstrating declining response. Although there is evidence that early treatment with high-efficacy DMTs offers better control over disease activity compared to low-efficacy DMTs; further consideration based on each patient's condition, is required to balance the safety and efficacy of either treatment.[144,145]

MS activity monitoring and diagnosis

Despite being described almost 150 years ago, the cause of MS still remains unknown and no one definitive test exist for MS. Diagnosis and disease activity monitoring is based on clinical examination, MRI, CSF studies and neurophysiology.[146]

Clinical marker

The clinical examination is the classical way to evaluate MS disease activity, progression, and frequency of relapses.[143] However, due to the heterogeneity of symptoms of MS, MRI of the CNS is necessary to support, supplement or even replace some clinical criteria. MRI examination can detect a new lesion in MS patients with or without clinical symptoms.[122] Therefore, MRI is a standard technology for the diagnosis

and monitoring of MS, by assessing focal lesions in the white matter and visualising the gray matter involvement and brain atrophy.[148,149]

The diagnosis of MS rests on proving dissemination of inflammatory CNS lesions in both space and time, and the exclusion of other disease processes. The incorporation of MRI into the McDonald Criteria for MS has facilitated the earlier diagnosis of the disease.[150]

Expanded disability status scale

The expanded disability status scale (EDSS) is the oldest clinical assessment tool in MS. It measures seven functional abilities of patients without concentration on cognitive changes. Although, EDSS represents a low sensitivity in clinical examination, is still listed as a quantifying disability in MS patients.[146]

There are significant numbers of new patients whose MRI findings are unclear or ambiguous due to white matter lesions of unknown etiology. Current monitoring of RRMS patients requires regular MRI scanning (every six months) which is associated with high cost and limited accessibility. Thus, an urgent need exists to identify blood-based biomarkers to confirm MS diagnosis and monitor disease activity. Using peripheral blood for diagnostic tests is a non-invasive and cost-effective method. However, identifying a reliable panel of biomarkers in MS has been extremely challenging with regard to obtaining high sensitivity and specificity and also reproducibility upon validation.[151] Many potential biomarkers have been proposed, including myelin-specific antibodies in serum,[152] neurofilament light chains,[153] and inflammatory markers in CSF,[151] however to date, no reliable biomarker has been introduced into routine clinical practice.

Biological markers

Biological markers (biomarkers) are measurable characteristics that indicate biological processes, pathogenic processes or pharmacologic responses to a therapeutic. Scientists have been utilising biomarkers to diagnose various diseases for more than 50 years. This process has expanded in the 21st century and more candidate biomarkers have been generated using high-throughput technologies such as –omics. Nowadays the importance of valid biomarkers in molecular diagnosis is undeniable.[154] Different molecules and cellular component can be used as biomarkers. In this study we are interested in examining some processes of intercellular communication to discover

reliable blood-based biomarkers for neurological diseases such as multiple sclerosis and glioblastoma.

Biomarker study in MS has a long history. Many molecules have been investigated, but no strong correlation has been detected. For instance, many studies have examined the level of proteins in the CSF or serum of MS patients, such as neurofilament, glial fibrillar acidic protein, Nogo and CD163. None of the studied proteins has sufficient specificity and sensitivity to be used as a marker, except for the IgG index and oligoclonal bands, which provide supportive evidence in MS diagnosis.[53] The focus of early epidemiology studies was on understanding the genetics of MS. The most reliable genetic link for MS susceptibility is the HLA-DRB1*15 haplotype.[130] However, progress in genome studies using whole genome admixture studies and next-generation sequencing methodologies have provided new molecular targets for mechanistic investigations, and biomarker discovery.[155] Bomprezzi et al. studied gene expression profiles in PBMC and observed a significant increase in the IL-7 receptor transcript in MS patients in comparison to healthy individuals.[156] Subsequent studies confirmed that the IL-7 gene is linked to increased MS susceptibility and also showed expression changes between RRMS patients in remission versus active relapse, and between SPMS and PPMS patients versus healthy individuals.[155,156] Later, Hecker et al. identified two gene transcripts, GPR3 and IL17RC, with increased expression in the blood of RRMS patients that had potential as biomarkers. Also, there have been many studies focused on determining the efficacy of drug treatment by monitoring changes in gene expression.[157] Overall, the result of these studies is mixed with very few overlapping gene transcripts between reports which might be attributable to a variety of factors such as the difference in MS patients subpopulations, sample sources, and experimental design.[155]

The role miRNAs in MS

MS is one of the most common neurological disorders in young adults. MS is a heterogeneous disease with unclear etiology and no definitive diagnostic test, which makes it a perfect candidate for biomarker study.[122,142]

In 2009, Junker *et al.*[96] and Du *et al.*[158] assessed the expression of miRNAs for the first time in cells derived from MS patients' lesions and blood samples respectively[96,158]. The first group[96] was interested in analysing miRNA profiles in active and chronic inactive MS lesions to find a link between miRNAs and specific dysregulated genes in MS and

also to identify novel miRNA targets. While Du et al. evaluated the expression of miRNAs in MS patients after treatment to determine a diagnosis biomarker and estimate drug responses.[158] Interestingly, both groups reported a particular dysregulated miRNA (miR-326)[96,158] with a regulatory function toward MS-associated proinflammatory lymphocytes Th17 differentiation.[159] Subsequently, several studies have profiled miRNAs from the brain, different cell populations[10] and peripheral blood mononuclear cells[160,161] from MS patients to identify potential biomarkers. The correlation between cellular dysregulated miRNAs and diseases, including MS, raised the question whether circulating miRNAs in body fluids are also correlated with diseases physiopathology.[162]

There is evidence of the existence of miRNAs and mRNAs outside cells. Valadi *et al.*, in 2007, detected them in cell culture.[42] This report was followed by parallel studies addressing the detection of cell-free miRNAs in the human biological fluid.[94,99,163,164] In 2008, Chim *et al.* demonstrated the presence of placental miRNAs in maternal plasma, which is readily detectable and is promising as a marker in the clinical setting.[164]

The elevation of miRNAs in serum[94] and plasma from cancer patients also confirmed that the expression levels of specific circulating miRNAs—tumour-derived miRNAs—could be related to the disease.[99] This finding was confirmed by a subsequent discovery of miRNAs derived from circulating blood cells under normal conditions, that could change in the disease state. Cell-free miRNAs in body fluids are reported being stable under harsh conditions (e.g., low/high PH, long-term storage and multiple freeze-thaw cycles) and resistant to RNase A digestion.[163,165] Thus circulating miRNA profiles in body fluids including serum, plasma and CSF, shed from different cells, reflect the biological status of the body and have been described as a novel biomarker.[165]

The first study that showed the involvement of the plasma circulating miRNAs as a potential prognostic and diagnostic biomarker for MS was reported in 2012 by Seigel *et al.* who compared a small group of MS patients to healthy individuals (four people in each group).[166] This initial report was followed by another plasma circulating miRNA study on a bigger number of RRMS[161] and SPMS patients[167]. Due to differences in the procedure for miRNA profiling (e.g., RNA extraction, miRNA quantification, and statistical analysis), the reported miRNAs represent minimal compatibility.[159,168] To

solve this issue, the standard method for sample handling shows a significant capacity in reducing technical variability between protocols.[109,168,169]

The fundamental approach to analysing miRNA profiles is based on a microarray analysis,[123] quantitative reverse transcriptase PCR,[166,167,170,171] and lately next-generation sequencing (NGS)[123] techniques. Keller *et al.* in 2014, for the first time, used NGS to assess dysregulated miRNAs in the whole blood of MS patients. They also confirmed identical regulation pattern for the eight miRNAs in the microarray analysis and NGS.[123] Although they demonstrated converging results for microarray and NGS methods, NGS is the better technique to detect almost all miRNAs in each sample with higher sensitivity as compared to microarray screens.[123,159,172] Nevertheless, microarrays and quantitative reverse transcriptase PCR have been employed in multiple studies to conduct miRNA profiling experiments as NGS is an expensive method and produces a large output which requires data processing pipelines to process and analyse the data.[96,123,159,167,173,174]

The stability of endogenous miRNAs in body fluids is a consequence of being loaded into high-density lipoprotein,[175] bounded by proteins,[176-178] or packed into extracellular vesicles[18,179]. However, although purified extracellular miRNAs from the culture medium of different cell lines and human body fluids have been demonstrated to be protected and delivered by high-density lipoproteins and proteins (e.g., Ago2 and nucleophosmin 1), so far no releasing mechanisms have been described to be associated with them.[175,176] Moreover, some studies highlighted that the majority of circulating miRNAs in body fluids are associated with Ago2 proteins rather than being packed in extracellular vesicles.[178] In fact, these miRNA-Ago2 complexes could drive from dead cells or degradated exosomes as they have been reported to be carried by exosomes.[162]

Role of exosomes in MS

Recently, exosomes and their cargo became a candidate for a potential biomarker, and many studies have attempted to determine their involvement in MS. Verderio *et al.* in 2011 for the first time reported the release of exosomes from microglia, the immune cells in the CNS, which is detectable with a high concentration upon brain inflammation in human and rodent. Thus, the presence and concentration of these exosomes in the CSF represent a biomarker of 'inflamed CNS', which may reflect a signature of the disease.[57] The following study on this topic has revealed that the number of exosomes in

the serum of RRMS patients, especially during relapsing, is higher in comparison to both progressive patients and healthy individuals. RRMS and SPMS/PPMS characterise distinctive clinical states. Relapsing-remitting patients are in an inflammatory state of disease, with a peak during relapses and decrease on remitting periods, while SPMS/PPMS patients present less inflammatory activity and turn to the progressive degenerative phase.[180] The association between the number of immune cell-derived exosomes and inflammatory conditions in autoimmune disease has been well established.[14] And the result of these two studies demonstrated the association between increased amount of exosomes and development of neuroinflammation.[57,180] Although it demonstrated exosomes' role in MS pathogenesis and their reflection of disease status, thus far these findings have not yielded a diagnostic or prognostic biomarker in MS.[53,180]

Several studies have demonstrated that exosomes released from different neuron cells could regulate the process of myelination in the CNS.[53] Myelin formation is a multistep process, which occurs during the development of the CNS and is controlled by a variety of factors. Oligodendrocyte precursor cells differentiate into mature oligodendrocytes with a capacity of producing myelin membrane component. Synthesised myelin lipids and proteins require signals received from the microenvironment or axon to induce myelin membrane trafficking and start to enwrap axons.[56] It is reported that oligodendrocyte-derived exosomes inhibit the growth of mature oligodendrocytes and myelin formation. Therefore, this study suggested that neurons coordinate myelin membrane biogenesis by controlling the secretion of oligodendrocytes -derived exosomes.[56,57,180] Conversely, exosomes released from mature oligodendrocytes in response to activation via specific receptors enhance myelin formation both at the development level and at the regeneration of damaged sheets.[53] Exosomes are also released from astrocytes possessing synapsinI, and dendritic cells. Astrocyte-derived exosomes promote nerve cell growth, survival, and differentiation, while exosomes secreted by dendritic cells stimulate oligodendrocyte precursor cells growth and promote the process of repair during demyelination.[53] Overall, exosomes released from different cells within the CNS play vital roles in myelination by influencing the growth and function of oligodendrocytes and neurons.[53]

Moreover, exosomes secreted from endothelial cells, lymphocytes, and platelets in response to pro-inflammatory stimulation induce disintegration of the BBB and

facilitate immune and myeloid cells transmigration.[53] So exosomes can reflect the status of cell activation and pathological changes.[181] Despite the low concentration of secreted exosomes, their information-rich contents including miRNA, and the remarkable stability of those contents within exosomes make exosomes a potential biomarker to indicate disease states.[18]

Glioma

Gliomas are tumours of the CNS that arise from glial cells.[182] The most common site of gliomas in the CNS is the brain, with rare metastasises beyond the CNS.[183] Gliomas comprise 30% of all brain tumours and 70–80% of all malignant brain tumours.[184] Although brain tumours are less prevalent than other cancer types, with an incidence rate of 5–6 per 100,000 people worldwide, they are the leading cause of cancer-related deaths in Australians under 40.[185,186] According to the World Health Organization (WHO), glioma tumours are classified as one of three types based on histological characteristics: astrocytoma (astrocytes), oligodendroglioma (oligodendrocytes), oliogoastrocytoma (mixed population of both), ependymoma (ependymal cells)[182,185,187] *(Figure1-8)*.

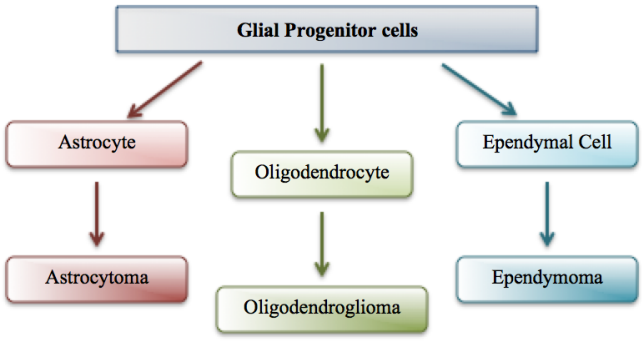


Figure 1-8 Classification of glioma tumours based on WHO

Gliomas comprise all primary CNS tumours of glial-cell origin. Glial cells include astrocytes, oligodendrocytes, and ependymal cells which develop astrocytoma, oligodendroglioma, ependymoma.

Each of these tumours is further graded according to their biological behavior to tumours with low (grades I and II; low-grade gliomas (LGGs)) and high malignancy (grades III and IV; high-grade gliomas (HGGs)).[188]

Gliomas also can be categorised based on their invasiveness into normal brain tissue to ‘diffuse gliomas’ and ‘circumscribed’. Diffuse gliomas not only have the ability to

infiltrate, but also over time recur and tend to progress to high-grade. Based on the WHO classification scheme astrocytoma can be graded into diffuse astrocytoma (Grade II), anaplastic astrocytoma (Grade III) and eventually glioblastoma (Grade IV) with a rapid clinical deterioration.[189] Grade I astrocytomas are potentially curable through surgery alone whilst grade II astrocytomas, even after surgery and radiotherapy, frequently return as higher grade gliomas.[190]

The leading risk factors for the development of glioma are largely unknown. Sequential and cumulative genetic alteration, exposure of the head and neck to ionizing radiation, aging and family history are the only risk factors indicated to date.[191,192] Although a family history of glioblastoma is rare, where it is present it, increases the risk of developing glioma up to two-fold.[191] Exposure to radiofrequency electromagnetic fields (EMFs) via cell phone is controversial, and despite a lot of attention as a potential risk factor for brain tumour development, it is not well supported as a risk factor.[192,193] Previous studies have indicated that dietary changes and supplementation provide a protective effect against glioma, although to date none has been proven effective and there has been no significant improvement in glioma survival rates for almost 30 years.[194]

The standard treatment for patients with glioma is usually surgery, followed by radiation therapy or combined radio- and chemotherapy depending on location, grade and type. While most benign tumours can be successfully treated by surgical excision, high-grade gliomas have poor prognoses.[191]

Molecular characterisation of gliomas

The previous WHO glioma classification was based on clinical, radiological and mainly histopathological characteristics of the tumour.[195] Histologic criteria for GBM diagnosis include hypercellularity, nuclear atypia, mitotic activity and either microvascular proliferation and/or tumour necrosis. However, the histologic features may not distinguish between different subtypes nor capture the molecular and cellular heterogeneity of brain tumour. Therefore, molecular signatures became candidates in classification criteria, diagnosis and treatment of malignancies.[189] For instance tumour protein 53 (TP53) mutation appears in the early stage of the development of an astrocytoma, whereas the loss or mutation of phosphatase and tensin homolog (PTEN)

and amplification of epidermal growth factor receptor (EGFR) occur in tumours that have progressed to a high grade.[196]

In 2016, for the first time, genotypic identification of tumour pathology has become a feature of the WHO classification of CNS tumours.[197] Thus, GBM is further classified based on somatic mutations of the metabolic enzyme isocitrate dehydrogenase isoforms (IDH1/2) into IDH^{WT} and IDH^{MUT}.[196,198] All reported mutation for IDH1 and IDH2 is located at codon 132 and 172 respectively.[198] IDH^{WT} type tumours account for 10% of GBM known as primary glioblastomas without previous lesions. However, the mutation in IDH was detected in 90% of secondary glioblastomas as a definitive diagnostic molecular marker with higher reliability than clinical or pathological criteria.[198] It has been suggested that IDH gene might be mutated after formation of the low-grade glioma to drive the progression of the disease to secondary glioblastoma.[196]

IDH-mutant glioblastoma is associated with an earlier diagnosis and better prognosis, with a median survival time of 27.1 months.[199] IDH genes encode a metabolic (isocitrate dehydrogenase) enzyme that plays a vital role in the citric acid cycle. Wild-type IDH1 catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate to produce nicotinamide adenine dinucleotide phosphate.[200] Mutation in either IDH1/2 reduces enzymatic activity[196] which results in decreasing nicotinamide adenine dinucleotide phosphate levels and produces an altered metabolite instead of nicotinamide adenine dinucleotide phosphate, 2-hydroxyglutarate[201]. Tumours harbouring IDH1/2 mutations in GBM have better prognosis in comparison to wildtype malignancies.[201,202] Therefore, IDH^{MUT} GBMs are sensitive to therapy and prolonged survival.

EGFR gene is also over–expressed in up to 57% of GBM tumours.[203] The majority of GBMs with EGFR amplification also carry the most common EGFR mutant gene, EGFRvIII.[204] EGFR dysregulation, especially EGFR amplification, promotes cell growth and division and is associated with an unfavourable prognosis.[205] The tumourigenic role for EGFR has made it a valuable target for therapeutic intervention.[206]

TP53 gene at chromosome 17p13.1 plays the guardian role in the genome that activates DNA repair or induces cell death in response to cellular stress.[207] TP53 network is also implicated in the tumourigenesis. TP53 is activated in 60% of early stage gliomas

(grade II & III astrocytoma) and found more frequently in secondary GBMs (>60%) than primary GBMs (~10%).[208]

Chromosomal arm deletions are also associated with gliomas. For instance, codeletion of chromosome 1p and 19q (1p/19q codel) is of interest as it raises the survival rate in oligodendroglioma.[209] Other low grade, IDH mutant tumours in the absence of 1p/19q codel contain TP53 mutation and ATRX inactivation *(Figure1-9)*.[198]

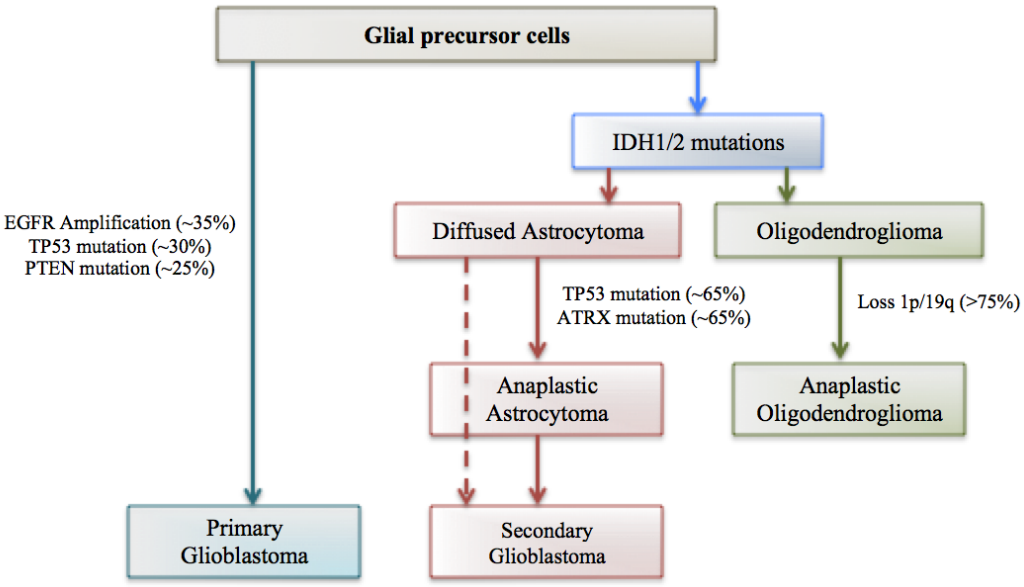


Figure 1-9 Genetic pathways to primary and secondary glioblastoma

Primary and secondary glioblastomas are histologically largely indistinguishable, however, mutations in IDH1/2 occur in early tumourigenesis and persist during progression to secondary glioblastoma. Cells with IDH1/2 mutations that subsequently acquire TP53 and ATRX mutations develop astrocytomas, whereas oligodendrogliomas shows acquisition of 1p/19q loss in cells with IDH1/2 mutations.

In low-grade gliomas, IDH1/2 mutations occur before TP53 mutation or 1p/19q loss as an early event in tumourigenesis.

Glioblastoma multiforme

Glioblastoma multiforme (GBM) is a grade IV astrocytoma. GBM is the most frequent and aggressive form of primary malignant brain tumour in adults, with a rising incidence over the last 20 years; notably in Australia.[182,189,191,210] An older population and improvements in imaging and diagnostic techniques are responsible in part, for the increase in GBM diagnosis; however, the influence of other factors remains to be

determined.[211] Unfortunately, GBM carries exceedingly poor prognosis with a median survival of 15 months, and most of the tumours recur locally.[191,212]

The high mortality rate of GBM and its poor response to conventional chemotherapy and radiation attest to its invasive nature. Therefore, new diagnostic approaches are required for better understanding GBM invasion and its molecular processes.[182]

GBM subtypes

The Cancer Genome Atlas (TCGA) research network utilised a comprehensive study on GBM to examine the molecular characteristics and classification of these heterogeneous tumours.[203] The TCGA study demonstrated that GBM might acquire or lose chromosomes and carry amplifications, mutations or deletions. These characteristics have provided details on the tumourigenesis of glioblastoma and potential targets for new therapeutic approaches. The TCGA has now further defined four subtypes for GBM: classical, mesenchymal, proneural and neural.[182,203] Based on the new classification each subtype demonstrates particular mutations. The characteristic of each subtype is listed in *Table 1-1*.

Table 1-1 Characteristic of glioblastoma subtypes

Subtype	Mutations	Clinical features
Classical	EGFR amplification, PTEN null, CDKN2A null, TP53 wild-type	Aggressive treatment significantly improved survival rates
Mesenchymal	cMET amplification, PTEN null, CDKN2A null, NF1 mutated, mesenchymal markers (CHI3LI)	Aggressive treatment significantly improved survival rates
Proneural	PDGFR amplification, PTEN null, CDKN2A null, IDH1 mutated, TP53 mutated, proneural development genes (SOX)	Some benefits to aggressive treatment
Neural	Neural markers (NEFL, GABARA1, SYT1 and SLC12A5)	Younger patients gain no survival benefit from aggressive treatment

Abbreviations: EGFR, epidermal growth factor receptor, PTEN, phosphatase and tensin homolog, CDKN2A, cyclin-dependent kinase inhibitor 2A, TP53, tumour protein p53, cMET, hepatocyte growth factor receptor, NF1, neurofibromin, CHI3LI, chitinase-3-like protein, PDGFR, platelet derived growth factor receptor, IDH1, isocitrate dehydrogenase 1, SOX, Sry-related high mobility box, NEFL, neurofilament like polypeptide, GABARA1, gamma-aminobutyric acid type A receptor alpha 1 subunit, SYT1, synaptotagmin 1, SLCI2A5, solute carrier family 12 member 5.

GBM pathophysiology

GBM has a unique pathophysiology compared to other gliomas with high degree of aggression and malignancy, which allows the tumour to spread out quickly to the point where even total hemispherectomy has failed to be curative.[210] The infiltrative growth of GBMs is a major challenge to managing their development and recurrence.[182] GBMs are invasive due to an extensive vascular system, disruption in the integrity of the BBB, high migration rate of tumour cells along vasculature, and GBM cells' potential to release proteases and degrade the extra-cellular matrix.[187]

Furthermore, GBMs are extremely heterogeneous, composed of many different cell types. Cancer stem cells have emerged as a possible sub-class of cells within tumours.

In general, a tumour microenvironment consists of a variety of cells including the cancer cells, cancer stem cells and endothelial cells.[213] GBM stem-like cells play a putative role in GBM aggressiveness and invasion[194] and are a recent focus in GBMs pathogenesis and potential treatment[214]. They have stem-like properties such as self-renewal and multi-lineage differentiation and as such are capable of repopulating the tumour after therapy, resulting in high rates of tumour recurrence and treatment failure.[214]

On a cellular level GBM cells produce actin-rich protrusions of the plasma membrane with small punctuate finger like projections in contact with the extra-cellular matrix, named invadopodia. Invadopodia display proteolytic activity and actin polymerisation.[215] Their function includes degradation of the extra-cellular matrix and facilitation of invasion and metastases in malignant tumours.

Treatment

The current standard of care for GBM, known as the Stupp protocol, consists of maximal surgical resection followed by radiotherapy and the chemotherapeutic drug temozolomide. The aim of surgery is maximal safe resection of the infiltrative tumour; however, resection is often incomplete, tumour recurrence inevitable, and the median overall survival following surgery, radiotherapy and chemotherapy is just over one year.[216] After surgery, radiotherapy causes DNA damage to the remaining GBM cells and a marginal increase in the survival rate of GBM patients. The addition of temozolomide, with a capacity to cross the BBB, to surgery and radiotherapy is associated with a significant improvement in overall survival of 15 months.[216]

Temozolomide is an oral DNA-alkylating agent currently used as first-line therapy for GBM treatment that adds methyl groups to DNA.[217] Temozolomide efficacy can be predicted by O^6-methylguanine-DNA-methyltransferase (MGMT) methylation.[217] Methylated MGMT is inactive and allows the temozolomide to damage the DNA and kill the tumour cells, however unmethylated MGMT actively repairs the DNA damage after temozolomide treatment.[218] As such patients with unmethylated MGMT promoters have a lower median survival time, 12.7 months, compared to 21.7 months for patients with methylated MGMT (inactive).[218]

The diffuse infiltrative nature of GBM makes it challenging to treat. There is hope that more molecular characterisations of GBM will open up new avenues for treatment and monitoring.

The role of exosomes and miRNAs in gliomas

Biomarkers are measurable indicators with high diagnostic and prognostic value that can also monitor physiological response to a therapeutic intervention. Selective biomarkers can detect tumours at an early stage, leading to better chances of recovery and survival for these patients over those with more advanced neoplasma at the time of diagnosis.[219] As described in the previous sections, biomarker status such as IDH1/2 mutations and chromosomal co-deletion of 1p/19q has changed the traditional classification of some gliomas' subtypes.

miRNAs from a family of small non-coding RNA have emerged as powerful platforms for tumour-forming processes[220] by playing a role in cell cycle regulation, cell proliferation, apoptosis, invasion, and angiogenesis[221]. Therefore miRNAs can be considered as cancer biomarkers. There is evidence that miRNAs are integrally involved in GBM oncogenic signaling and have the potential to serve a disease biomarker.[219-221] A previous study on miRNA expression in glioma tissue implicated some miRNAs as being involved in tumour formation and propagation.[222] The researchers investigated the genome-wide miRNA expression pattern using miRNA microarray assay and deep sequencing. They reported 97 dysregulated miRNAs in glioblastoma compared to the healthy brain and 22 in adjacent brain samples in comparison to normal brain. The only outstanding miRNA was miR-625 which showed exclusive downregulation within the borders of tumours, but not in GBM samples.[222] It has been reported that differential expression of miRNA-625 in gastric acid targets integrin-like kinase gene product and increases invasion and migration.[223]

Hypothesis and aims

As detailed above there are a variety of molecules that can indicate the presence and progression of a disease or its therapeutic response. Exosomes are released by almost all cell types, carry a selective package of cargo and are enriched in miRNAs with a regulatory post-transcriptional function.

The overarching HYPOTHESIS of this study is that exosomal-associated microRNAs present in the peripheral blood are sensitive, specific and robust biomarkers of neurological disease.

The specific AIMS are as follows:

1. Establish and validate a robust protocol for isolating exosomes and exosomal miRNAs from serum samples.
2. Use the methods above for a pilot study to examine MS patients and their healthy matched control to determine if serum exosomal miRNAs are able to distinguish MS patients from healthy individuals, and MS patients in different disease phases.
3. Use the methods in (1) above to examine the utility of serum exosomal miRNAs in monitoring response to the common MS drug Fingolimod in known RRMS patients.
4. Use the methods in (1) above to examine the utility of serum exosomal miRNAs in the diagnosis of another neurological disease – glioblastoma.

Chapter 2:
Exosomal microRNA signatures in MS reflect disease status

This chapter contains the original research article 'Exosomal microRNA signatures in multiple sclerosis reflect disease status' which has been published in the *Journal of Scientific Reports*, volume 7, article number: 14293 (2017).

Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. There is currently no single definitive test for MS. Circulating exosomes represent promising candidate biomarkers for a host of human diseases. Exosomes contain RNA, DNA, and proteins, can cross the blood-brain barrier, and are secreted from almost all cell types including cells of the central nervous system (CNS). We hypothesized that serum exosomal microRNAs (miRNAs) could present a useful blood-based assay for MS disease detection and monitoring. Exosome-associated miRNAs in serum samples from MS patients (n=25) and matched HCs (HC) (n=11) were profiled using small RNA next generation sequencing. We identified differentially expressed exosomal miRNAs in both relapsing-remitting multiple sclerosis (RRMS) (miR-15b-5p, miR-451a, miR-30b-5p, miR-342-3p) and progressive MS patient sera (miR-127-3p, miR-370-3p, miR-409-3p, miR-432-5p) in relation to controls. Critically, we identified a group of nine miRNAs (miR-15b-5p, miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR-432-5p) that distinguished relapsing-remitting from progressive disease. Eight out of nine miRNAs were validated in an independent group (n=11) of progressive MS cases. This is the first demonstration that miRNAs associated with circulating exosomes are informative biomarkers not only for the diagnosis of MS, but in predicting disease subtype with a high degree of accuracy.

Introduction

MS is the most common cause of neurologic disability in young adults.[224] MS is characterised by inflammation, demyelination, and neuro-axonal injury in the CNS, leading to progressive, long-term disability.[224] The clinical phenotypes of MS include RRMS, and progressive forms: secondary progressive multiple sclerosis (SPMS) and PPMS.[142] RRMS is the most prevalent MS subtype, comprising over 70% of cases. Within 10 to 15 years of disease onset, the majority of patients with RRMS will transition to SPMS, a phase of the disease defined by gradual clinical worsening that does not respond to any available treatment. PPMS is clinically indistinguishable from SPMS, except that it manifests *de novo*, without a preceding relapsing-remitting phase.

Currently there is no one definitive test for MS assessment; diagnosis and disease monitoring relies on multiple clinical parameters including clinical examination, magnetic resonance imaging, cerebrospinal fluid assessment, and electrophysiology.[136] Such investigations are not only costly over the protracted disease course; they also have limited utility in distinguishing active RRMS from progressive disease.[142,225]

Here we have assessed the utility of miRNA within serum exosomes as biomarkers of MS disease. MiRNA are small (18–25 nt) noncoding RNA with post-transcriptional gene regulatory function.[226] Exosomes are membrane-bound vesicles shed by almost all cell types, and packed with small regulatory RNAs such as miRNA.[62] In many inflammatory diseases there is a significant increase in circulating exosome concentration.[227,228] Given that exosomes can cross the blood-brain barrier,[8,9] it is thus likely that at least some of the circulating exosomes in MS patients are derived from affected CNS cells or the associated inflammatory milieu.

We hypothesized that physiological changes associated with MS and its progression are reflected in differences in serum exosomal miRNAs. Using next-generation sequencing and integrative bioinformatics we found that serum exosome miRNA profiles can not only distinguish MS from HCs, but also distinguish RRMS from progressive forms of the disease with high accuracy.

Materials and methods

Participants

All patients attended the Royal Prince Alfred Hospital MS Clinic at the Brain and Mind Centre, The University of Sydney. The study was ethically approved by the RPA Hospital Human Research Ethics Committee (#X13-0264), and all patients provided written informed consent. All methods were performed in accordance with the relevant guidelines and regulations. MS was diagnosed according to the revised McDonald criteria,[149] and SPMS patients were differentiated from the other clinical phenotypes (RRMS and PPMS) using the definitions offered by Lublin *et al*.[142]

Sample collection and preparation

A 20 ml blood sample was obtained from each participant's using venepuncture with a 23-gauge butterfly needle. Blood was collected in three BD Vacutainer SST II Advance Serum-gel 7.5-ml Tubes (BD Vacutainer®, USA). Serum-gel tubes were left at room temperature for 30 minutes for coagulation, and then centrifuged at 1,800 g for 10 minutes. The resulting serum was transferred into 15 ml tubes and centrifuged at 3,000 g for 20 minutes to remove any cellular debris. The serum sample was aliquoted into 2 ml microcentrifuge tubes with O-rings (Interlab®, New Zealand), immediately snap-frozen in liquid nitrogen and stored at -80°C. All serum-gel tubes were processed within two hours of collection.

Exosome purification and characterisation

Serum (1 ml from each individual) was treated with RNase A at 37°C for 10 minutes (100 ng/ml, Qiagen, Australia) before exosome purification. The treated serum then underwent size exclusion chromatography (qEV iZON Science) by being overlaid on qEV size exclusion columns followed by elution with 5 ml freshly filtered PBS. Ten fractions of 500 µl each were collected and analysed with nanoparticle tracking analysis (NanoSight, Amesbury, UK). Fractions 8, 9, and 10 were pooled and stored at -80 °C for downstream analysis.

Western immunoblotting

Purified exosomes were resuspended with 4X sodium dodecyl sulfate loading buffer and heated at 95 °C for 5 minutes to lyse. Samples were resolved on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membrane at 400 mA for one hour using Criterion™ Blotter (BioRad, Hercules, CA, USA). Membranes were blocked in TBS-T containing 5% skim milk (w/v) followed by overnight incubation at 4°C with a primary antibody (CD63, Abcam, ab193349, CD81, ProSci, 5195, Alix, Cell Signaling 21715). Membranes were washed with TBS-T (triplicate, 5 minutes) and incubated with a secondary antibody (conjugated to horse-radish peroxidase) for one hour at room temperature followed by three more TBS-T washing steps. Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Inc.) detection reagent and imaged manually using X-ray film.

Transmission electron microscopy

10 µl of purified exosomes were loaded onto carbon-coated, 200 mesh Cu formvar grids (#GSCU200C; ProSciTech Pty Ltd, QLD, Australia) and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were negatively stained with 2% uranyl acetate for two minutes and dried overnight. Then samples were visualised at 40,000 X magnification on a Philips CM10 Biofilter TEM (FEI Company, OR, USA) equipped with an AMT camera system (Advanced Microscopy Techniques, Corp., MA, USA) at an acceleration voltage of 80 kV.

RNA extraction

Purified exosomes were processed for RNA extraction using the Plasma/Serum Circulating & Exosomal RNA Purification Mini Kit (Norgen Biotek, Cat. 51000) according to the manufacturers protocol. To check the yield, quality and size of extracted total RNA we analysed samples with an Agilent 2100 Bioanalyser (Agilent Technologies, United States) on a Eukaryote Total RNA chip.

Small RNA sequencing

Sequencing libraries were constructed from exosome RNA using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (BioLabs, New England) according to the manufacturer's instructions. Yield and size distribution of resultant libraries were validated using Agilent 2100 Bioanalyser on a high-sensitivity DNA assay (Agilent Technologies, United States). Libraries were then pooled with an equal proportion for multiplexed sequencing on Illumina HiSeq2000 System at the Ramaciotti Centre for Genomics.

Data pre-processing and differential expression analysis

Data pre-processing was performed using a pipeline comprising of adapter trimming (cutadapt), followed by genome alignment to human genome hg 19 using Bowtie (18bp seed, 1 error in seed, quality score sum of mismatches <70). Where multiple best strata alignments existed, tags were randomly assigned to one of those coordinates. Tags were annotated against mirBase 20, and filtered for at most one base error within the tag. Counts for each miRNA were tabulated and adjusted to counts per million miRNAs passing the mismatch filter. Samples with low miRNA read counts (<50,000) and miRNAs with low abundance (<100 read counts across more than 50% of samples) were removed (two RRMS and three S/PPMS samples). Differential expression analysis was performed using three different statistical hypothesis tests including a non-parametric two-sample Wilcoxon test and two parametric tests: Student's t-test, and an exact test (implemented in Bioconductor EdgeR) which tests for differences between the means of two groups of negative-binomially distributed counts. Data pre-processing and differential expression analysis were performed using Bioconductor and R statistical packages.

Univariate analysis

We performed logistic regression (LR) and receiver operator characteristic (ROC) analysis to assess the predictive power of individual miRNAs between the two groups of interest. LR was used to identify linear predictive models with each miRNA as the univariate predictor. The quality of each model was depicted by the

corresponding ROC curve, which plots the true positive rate (i.e., sensitivity) against the false-positive rate (i.e., 1-specificity). The area under the ROC curve (AUC) was then computed as a measure of how well each LR model can distinguish between two diagnostic groups. We then used leave-one-out cross-validation (LOO-CV) to estimate the prediction errors of the LR models. LOO-CV learns the model on all samples except one, and tests the learnt model on the left-out sample. The process is repeated for each sample and the error rate is the proportion of misclassified samples. Overall, cross validation is a powerful model validation technique for assessing how the results of a statistical analysis can be generalized to an independent dataset.[229] These analyses were performed using R stats (glm) and boot (cv.glm) packages.

Multivariate analysis

The predictive power of multiple miRNAs as disease multivariate signatures was assessed using random forest (RF) modelling. RF modelling is an ensemble learning method for classification/regression that operate by constructing a multitude of decision trees at training time in order to correct for the overfitting problem.[230] We used the R 'RandomForest' package which reports out-of-bag (OOB) errors as an unbiased estimate of the test set prediction error. The model computes the 'importance' of each predictor by permuting OOB data; that is, for each tree the misclassification error rate on the OOB portion of the data is recorded. The same procedure is done after permuting each predictor variable. The difference between the two are then averaged over all trees, and normalized by the standard deviation of the differences.

Results

Serum exosomes carry a unique miRNA signature

Patient blood was collected at the time of clinical consultation and pre-processed as detailed in the Methods. Exosomes were isolated from 1 ml of serum by SEC. Prior to exosome isolation, serum samples were treated with RNaseA to remove any unprotected circulating RNA. SEC fractions containing vesicles were pooled (fractions 8, 9, and 10; see Materials and Methods) and analysed by nanoparticle

tracking analysis *(Figure 2-1A)* and transmission electron microscopy *(Figure 2-1B)*. These analyses revealed a population of nanovesicles with a predominant size of 95 nm and cup-shaped morphology typical of exosomes. Western blotting of protein extracts for CD61, CD83 and Alix, confirmed that the particles isolated expressed all three characteristic exosome markers *(Figure 2-1C).* RNA extraction from each sample yielded the typical RNA profile for exosomes, with the absence ribosomal RNA and enrichment of small (<200nt) RNA species *(Figure 2-1D).* Small RNA libraries were constructed from the exosomal RNA and sequenced to yield on average about 10 million reads per sample.

To confirm that our protocols were selecting for small RNAs protected by association with exosomes, we compared miRNA profiles between four samples with and without RNAse pre-treatment. This identified 62 miRNAs whose relative expression differed significantly by at least 2-fold *(Figure 2-1E).* This demonstrates that serum exosomes carry a distinct pool of protected miRNA that can be interrogated in MS diagnosis and progression.

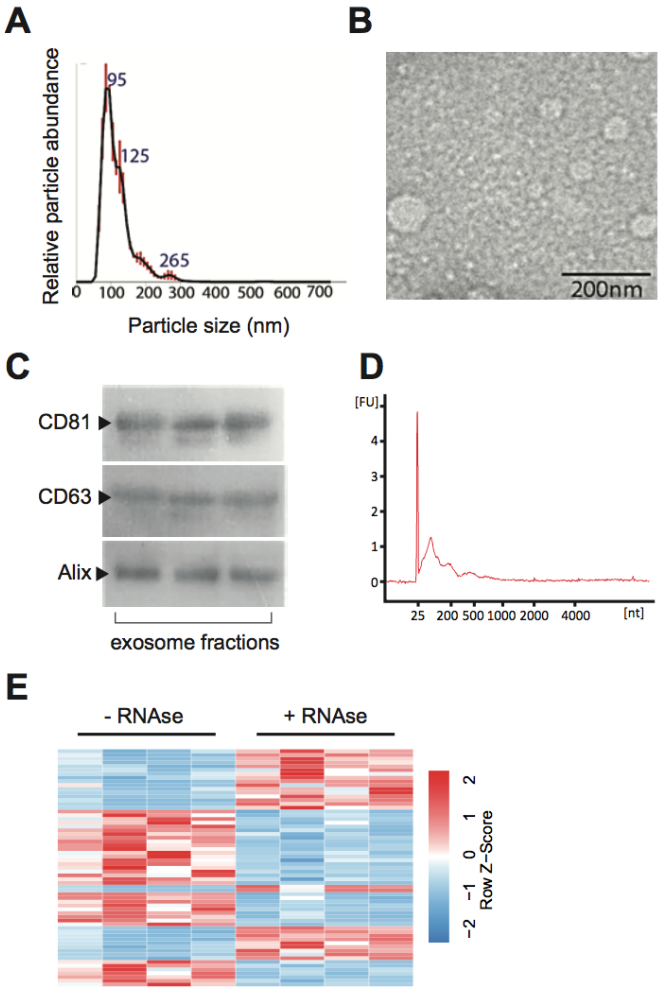


Figure 2-1 Identification and characterisation of serum exosomes

A) Size distribution of serum exosomes purified by size exclusion chromatography as analysed by nanoparticle tracking analysis. B) Transmission electron micrograph of serum exosomes demonstrates small vesicles with sizes ranging from 60–110 nm in diameter. C) Western blotting for exosome-associated proteins CD63, CD81 and Alix in three separate patient samples. D) Bioanalyser trace of RNA extracted from serum exosomes reveals a predominant population of small RNAs without ribosomal RNA. E) Hierarchical clustering of differentially expressed miRNAs shows that RNaseA treatment of serum results in unique miRNA population, (p-value ≤ 0.05 and fold change ≥ 2).

Exosomal miRNAs are dysregulated in MS patients and differentially expressed between disease subtypes

Twenty-five unrelated individuals with a diagnosis of MS (relapsing-remitting n=14, progressive MS n =11 (SPMS n=7, PPMS n=4)) and 11 healthy individuals were

studied. A second, independent set of progressive cases (*n*=11) was then analysed; participant demographic and clinical characteristics are outlined in *Table 2-1*.

Table 2-1 Characterisation of participants in this study

Clinical characteristics	RRMS (*n* = 14)	S/PPMS (Dis.) (*n* = 11)	HC (*n* = 11)	S/PPMS (Val.) (*n* = 12)
Age (mean ± SD)	42.5 (9.04)	53.4 (7.2)	40.3 (13.3)	53.1 (8.7)
Age of onset (± SD)	35.6 (7.28)	38.4 (8.5)	NA	33.9 (9.4)
Gender (F/M)	10/4	5/6	9/2	11/1
Disease duration in year (± SD)	6.9 (7.1)	15 (9.4)	NA	19.2 (6.1)
Treatment (Y/N)	6/8	4/7	0/11	8/4
EDSS (± SD)	1.5 (1.0)	5.3 (1.6)	NA	6 (1.1)

Abbreviations: RRMS, relapsing-remitting multiple sclerosis; S/PPMS, secondary/primary progressive multiple sclerosis; HC, health control; Dis., discovery set; Val., validation set; EDSS, expanded disability status score; NA, not applicable.

The healthy control (HC) cohort was selected to match for age and gender to the RRMS group. While progressive MS is associated with older age and different gender ratio compared to RRMS,[231] Pearson correlation demonstrates that age, gender and treatment did not correlate with the expression profiles of the identified miRNAs *(Table 2-2).* Also, these clinical characteristics when incorporated to the multivariate modeling have minimal contributions to the model's prediction accuracy *(Figure 2-2).*

Table 2-2 Correlation of individual miRNA with demographic variables

miR-	Age	Gender	Treatment
23a-3p	0.099	0.347	0.017
374a-5p	0.013	0.295	0.000
433-3p	0.411	0.225	0.330
223-3p	0.124	0.347	0.000
15b-5p	0.095	0.347	0.052
485-3p	0.559	0.295	0.277
432-5p	0.348	0.277	0.382
30b-5p	0.096	0.416	0.000
342-3p	0.126	0.468	0.069
Average	**0.212**	**0.322**	**0.168**

Pearson correlation of age, gender and treatment with individual miRNAs show that these clinical variables are not correlated with the expression profiles of identified miRNAs across RRMS and S/PPMS samples (similar results attained using ranked-based correlation techniques, namely, Spearman or Kendall).

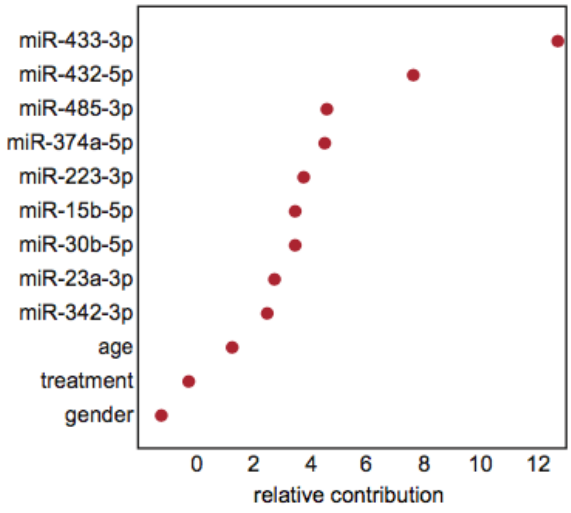


Figure 2-2 Relative contribution of each variable

Age, gender and treatment were incorporated as predictors into the random forest nonlinear and multivariate model. The importance plot demonstrates the minimal contribution of these variables in predicting MS subtypes and illustrates the predictive power of signature miRNAs independent of these clinical characteristics.

We employed three statistical approaches (Student's t-test, Fisher's exact, Wilcoxon rank sum) to identify differential expression of miRNAs between HCs, RRMS and progressive MS. miRNAs were identified as differentially expressed if they met a fold-change ≥2, and *p*-value ≤0.05 in at least two of the three statistical tests. Using this strategy, we identified four significantly dysregulated miRNAs between HCs and RRMS patients, and a further four between HCs and MS patients with progressive disease (SPMS/PPMS; *Table 2-3).* These represent miRNAs that have the potential to be exploited as blood-based diagnostic markers.

We also compared miRNA profiles between the two clinically distinct MS subtypes, RRMS and progressive MS. Here we found nine miRNAs that were significantly differentially expressed between the two subtypes *(Table 2-3).* Importantly, *in silico* validation by leave-one-out cross validation (LOO-CV) correctly identified the test sample on average 80% of the time (range 77–86%; *Table 2-3).*

Table 2-3 Significantly dysregulated miRNAs across all group comparisons

	MiRs	CPM (B)	CPM (A)	FC	t-test	Exact test	Wil-coxon	Error rate
Control (A) vs. RRMS (B)	15b-5p	314.2	145.9	2.15	0.045	0.002	0.05	0.23
	451a	39,592	19,114.8	2.07	0.009	0.0003	0.005	0.20
	30b-5p	673.48	246.55	2.73	0.06	0.0004	0.026	0.21
	342-3p	329.58	132.79	2.48	0.05	0.0002	0.008	0.21
Control (A) vs. S/PPMS (B)	127-3P	1,715.1	752.4	2.28	0.007	0.001	0.003	0.17
	370-3p	707.66	321.77	2.2	0.008	0.002	0.007	0.18
	409-3p	2,893.2	1,385.2	2.08	0.005	0.002	0.002	0.17
	432-5p	682.88	308.67	2.2	0.002	0.001	0.003	0.16
S/PPMS (A) vs. RRMS (B)	15b-5p	314.2	135.73	2.31	0.04	0.008	0.05	0.23
	23-3p	1,116.69	506.34	2.2	0.04	0.005	0.025	0.21
	223-3p	2,646.92	934.56	2.8	0.026	0.002	0.047	0.22
	74a-5p	328.22	159.26	2.06	0.02	0.009	0.038	0.22
	30b-5p	673.48	219.50	3.06	0.05	0.001	0.015	0.2
	433-3p	195.47	414	0.47	0.003	0.0027	0.0007	0.14
	485-3p	295.44	618.44	0.47	0.0056	0.002	0.004	0.17
	342-3p	329.58	130	2.53	0.05	0.0016	0.02	0.22
	432-5p	329.88	682.88	0.48	0.004	0.006	0.005	0.19

Abbreviations: CPM, miRNA counts per million; FC, fold change; RRMS, relapsing-remitting multiple sclerosis; S/PPMS, secondary/primary progressive multiple sclerosis; HC, healthy control; EDSS, expanded disability status score; NA, not applicable; error rate, estimated by leave-one-out cross validation.

An independent validation set of 11 new progressive MS samples was then sequenced and analysed using the same methods. Differential expression analysis between this new group and HCs confirmed that three of the four original miRNAs (miR-370-3p, miR-409-3p, miR-432-5p) were significantly dysregulated. The fourth miRNA (miR-127-3p), while exhibiting close to two-fold change in expression between the groups, failed to reach statistical significance *(Table 2-4).* Differential expression analysis between the validation group and RRMS samples identified eight out of nine significantly dysregulated miRNAs as identified previously (miR-15b-5p,

miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR-432-5p) *(Table 2-4)*.

Table 2-4 Significantly dysregulated miRNAs using progressive MS validation set

	MiRs	CPM (B)	CPM (A)	FC	T-test	Exact test	Wil-coxon	Error rate
Control (A) vs S/PPMS (B)	127-3p	402	752	0.53	0.08	0.03	0.07	0.25
	370-3p*	625	322	1.94	0.05	0.17	0.04	0.24
	409-3p*	2585	1385	1.87	0.002	0.0002	0.003	0.19
	432-5p*	589	309	1.91	0.03	0.6	0.03	0.23
	15b-5p*	314	110	2.8	0.017	7E-08	0.0004	0.17
	223-3p*	2647	675	3.9	0.011	0.0004	0.0005	0.15
	23a-3p*	1116	557	2	0.047	0.6	0.015	0.20
S/PPMS (A) vs RRMS (B)	30b-5p*	673	90	7.5	0.014	2E-09	0.000001	0.00
	342-3p*	329	103	3.2	0.029	0.034	0.0007	0.17
	374a-5p*	328	188	1.7	0.033	6E-07	0.133	0.23
	432-5p*	329	589	0.5	0.051	0.0005	0.059	0.24
	433-3p*	195	492	0.4	0.006	1E-09	0.002	0.18
	485-3p	295	220	1.3	0.181	0.06	0.211	0.27

*MiRNAs whose p-value < 0.05 in at least two tests and FC ≥ 1.7 in either directions.
Abbreviations: c.f. *Table 2-3*.

Serum exosomal miRNAs reflect MS subtypes

We next examined the predictive power of each miRNA in our discovery sets using LR models in which the predictor was the individual miRNA expression profile. ROC curves were determined for each candidate miRNA, where the true positive rate (sensitivity) is plotted against the false positive rate (1 – specificity). AUC measures were ≥ 0.74 for each individual miRNA, for both RRMS and S/PPMS groups compared to HCs *(Figure 2-3)*; for RRMS compared to S/PPMS the AUC measurements were ≥ 0.76 *(Figure 2-4)*.

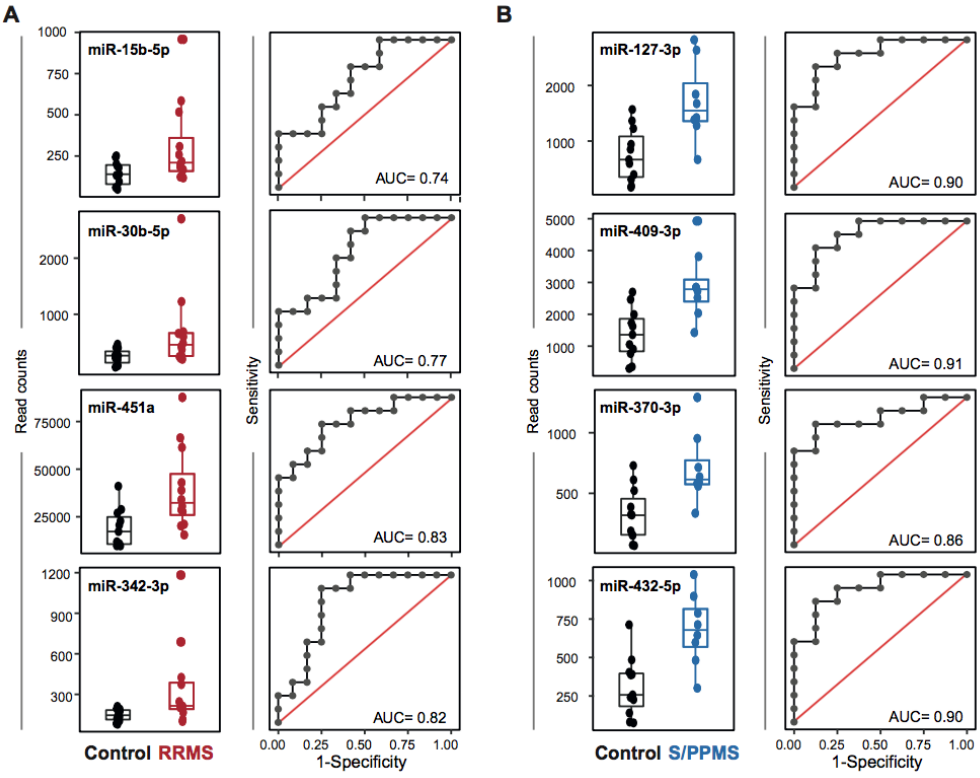


Figure 2-3 Differentially expressed miRNAs for control vs. RRMS or S/PPMS groups

Differentially expressed miRNA species were identified by Student's t-test, Fisher's exact test (EdgeR), and the Wilcoxon rank sum test for control versus RRMS (A) and control versus S/PPMS (B). MiRNAs with fold-change ≥ 2 and p-value ≤ 0.05 in at least two tests were identified as being differentially expressed. Left panels: Box-and-whisker plot for each miRNA species between the two groups (black box represents control group, red and blue boxes represent RRMS and S/PPMS respectively). Right panels: Logistic regression and receiver operator characteristic analysis performed on individual miRNAs to assess predictive power. LR was used to determine the linear model with the best discriminatory power between control and MS patient samples. The quality of this model was measured by the AUC displayed on each plot.

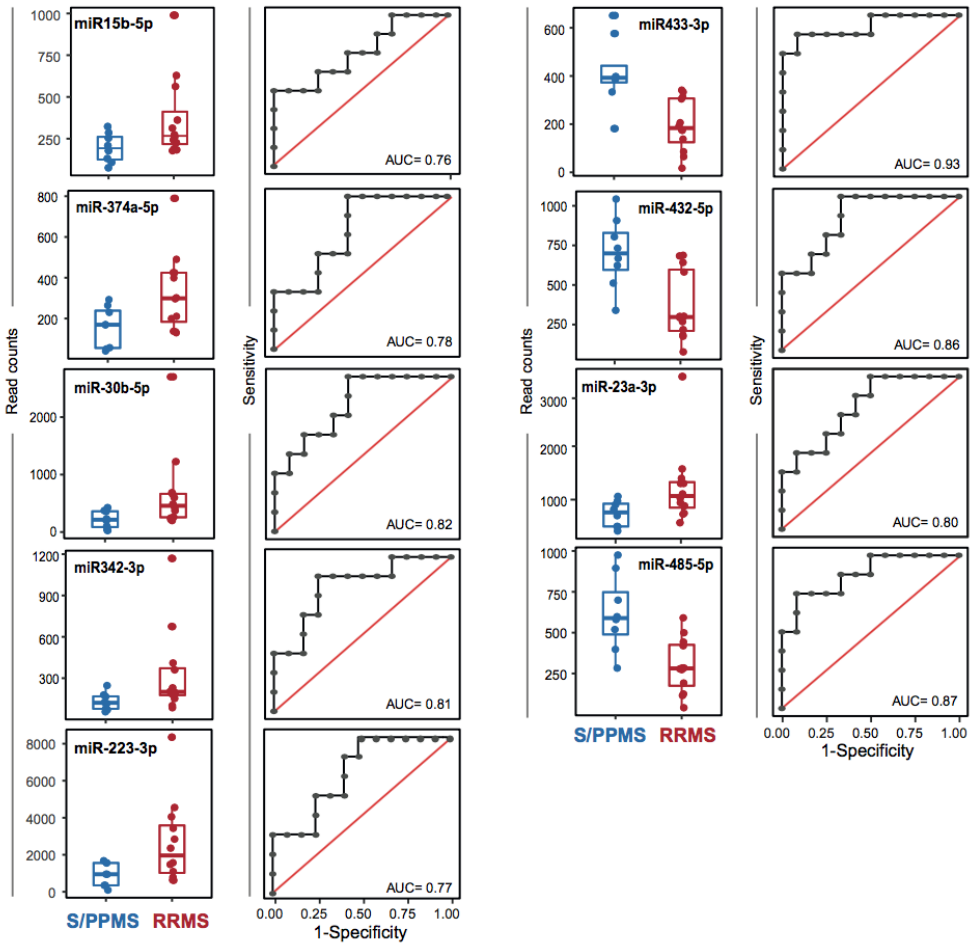


Figure 2-4 Differentially expressed miRNAs for RRMS vs. S/PPMS groups

Differentially expressed miRNA species were identified as per *Figure 2-3* above. **Left panels:** Box-and-whisker plot for each miRNA species between the two groups (red = RRMS group and blue represent S/PPMS group). **Right panels:** Logistic regression and receiver operator characteristic analysis of individual miRNAs to assess predictive power. Logistic regression was used to determine the linear model with the best discriminatory power between control and MS patient samples. The quality of this model was measured by the AUC displayed on each plot.

The relative importance of each miRNA in our discovery sets, when considered individually, was calculated using the random forest method and these shown in *Figure 2-5A*. Multivariate analyses using random forest were used to determine whether the combined expression patterns of multiple miRNAs could improve this predictive power. All possible miRNA combinations in each comparator group were trialed; the corresponding random forest multivariate models were then generated and OOB error rates estimated. Using these methods, we were able to achieve predictive power of 66% for RRMS and progressive MS versus controls. Strikingly

however, a combination of three or more miRNAs provided a predictive power of 95% for distinguishing RRMS from progressive MS *(Table 2-5* and *Figure 2-5B*).

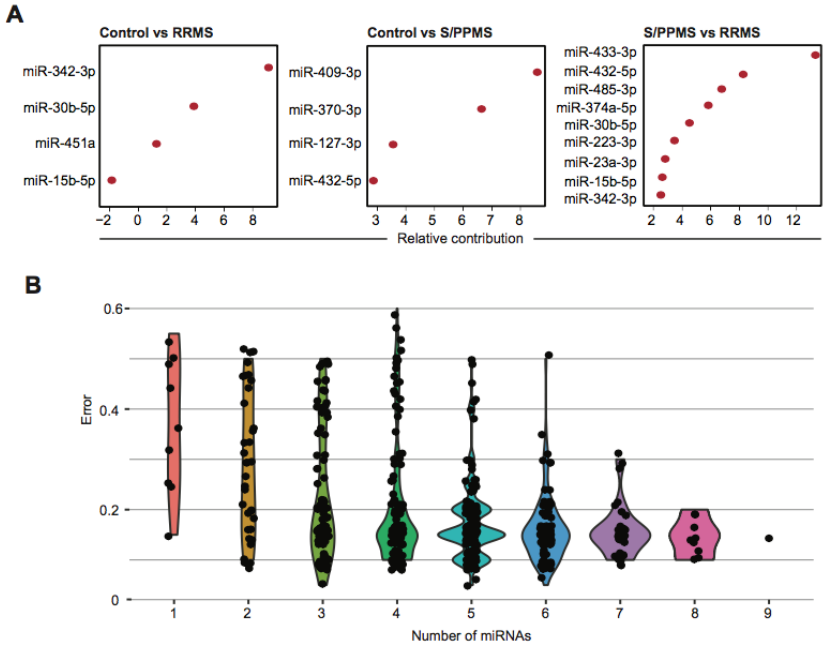


Figure 2-5 Random forest multivariate analysis

A) Significantly dysregulated miRNAs in each comparator group were ordered by the importance of contribution towards clinical classification as measured by random forest models. B) Random forest model was run using all possible combinations of dysregulated miRNAs to find combinations (i.e., signatures) with highest multivariate predictive power. Error rates of different combinations were stratified by the number of miRNAs (signature size) and their distributions were displayed as violin plots. This figure shows results achieved in RRMS vs SPMS/PPMS comparisons. Similar analyses were performed for other comparator groups and summarized in *Table 2-5*.

Table 2-5 miRNA combinations improve discriminatory power between relapsing-remitting (RRMS) and progressive (SPMS/PPMS) disease

# of miRNAs	miRNA composition	Error
9	miR-15b-5p, miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR-432-5p	0.15
6	miR-15b-5p, miR-23a-3p, miR-223-3p, miR-30b-5p, miR-485-3p, miR-432-5p	0.05
5	miR-23a-3p, miR-374a-5p, miR-30b-5p, miR-485-3p, miR-432-5p	0.05
5	miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-485-3p	0.05
3	miR-223-3p, miR-485-3p, miR-30b-5p	0.05

We then examined the accuracy of random forest analysis in predicting the status of the validation set of new progressive samples using the same miRNA signatures. In this new test set, the original nine miRNAs reported for RRMS vs S/PPMS could predict 11/11 progressive MS samples in the validation sets (i.e., class specific error rate = 0%).

Pathway analysis of dysregulated miRNAs

We performed functional analysis on targets of identified miRNAs. For each signature, we retrieved validated targets of miRNAs from three major miRNA-target datasets, miRecords,[232] miRTarBase[233] and TarBase[234] using the multiMiR R analysis package[235]. We then performed pathway overrepresentation analysis using KEGG pathways retrieved from the Molecular Signatures Database (MSigDB)-V 6.0.[236] Among top 5% of significantly enriched pathways (adjusted-*p*-value < 10E-5), we observed relevant pathways such as neurotrophin signalling pathway, focal adhesion, and T cell receptor signalling.

Discussion

In this study we have used unbiased high-throughput sequencing on RNA derived from serum exosome preparations in order to capture the complete profile of these miRNAs in patient sera. We used size exclusion chromatography for exosome isolation; a method that is known for high purity of exosome extracts as well as high reproducibility.[237] This method, coupled with RNAse treatment of extracts, allows interrogation of exosomal-associated miRNAs; a source of biomarkers distinct from free circulating miRNA. Machine-learning approaches on miRNAs were used to examine their individual and collective predictive powers to identify disease subtype in MS. The results from this study confirm that exosome-associated miRNAs represent unique and potentially powerful biomarkers for this common neurological disease.

We have identified dysregulated miRNAs that discriminate healthy individuals from RRMS or SPMS/PPMS patients with good predictive power. We also identified nine miRNAs that distinguish RRMS from SPMS/PPMS patients with a very high degree

of accuracy. A combination of just three miRNAs (miR-223-3p, miR-485-3p, miR-30b-5p) had a 95% accuracy rate of predicting disease progressive forms of MS from RRMS as identified by random forest analyses, suggesting that they may be useful clinical biomarkers. An independent validation set of progressive MS samples confirmed the reproducibility of our findings, and random forest analysis correctly categorised all samples in this new test set as progressive MS. To date, there are no clear clinical, imaging, or pathologic criteria to determine the point when RRMS converts to SPMS.[142] Our findings indicate that serum exosomal miRNA profiles may be a useful tool in assisting determination of this transition.

Some of the miRNAs we have identified have been previously implicated as circulating biomarkers in multiple sclerosis, namely miR-23a, miR-15b, miR-223, and miR-374a.[92,96,123,167,170,225,238-240] MiR-23a is involved in oligodendrocyte differentiation[241] and increases within active and chronic MS lesions.[96] Also, both miR-23a and miR-15b target the fibroblast growth factor-2 gene.[242] The fibroblast growth factor-2 is implicated in demyelination and remyelination, and there is some evidence that cerebrospinal fluid (CSF) the fibroblast growth factor-2 may be a useful marker of inflammation in MS.[243] MiR-223 is one of the few miRNAs that have been identified across several independent blood-based miRNA studies in MS,[225] and it targets the transcription factor STAT5 and other inflammatory regulators implicated in MS such as heat shock protein 90 and E2F.[244-246]

While several candidate miRNAs have been previously reported as potential MS biomarkers, the majority we have identified are novel. This likely reflects the unique constituent profile of exosomes versus free circulating miRNAs, and demonstrates that serum exosomal preparations represent a novel source of biomarkers. miR-451a was upregulated in RRMS patients compared to HCs; a miRNA previously reported as a regulator of oxidative stress with potential importance in a variety of neurodegenerative process.[247] We also identified miR-342-3p to be upregulated in RRMS patients; a miRNA especially enriched in microglia and dysregulated in Creutzfeldt-Jakob and Alzheimer's disease.[248-250] Both miR-342-3p and mir-30b-5p have been proposed as free circulating miRNA biomarkers in Alzheimer's and Parkinson's diseases,[27,28] and their association with MS in this study suggests that they may be more general markers of neuro-axonal injury. Pathway analysis of

transcripts known or predicted to be regulated by our candidate miRNA profiles yielded functional pathways highly relevant to MS disease pathogenesis such as neurotrophin signaling,[251] focal adhesion[252] and T cell receptor signalling pathways[253].

Small RNA analysis from biological fluids, including exosomal miRNAs, are subject to a variety of pre-analytical variables such as sample collection and processing methods, as well as differences in coagulation processes of serum and plasma.[169,254] This likely contributes to the only partially overlapping 'free circulating' miRNA profiles reported in different studies of MS to date.[92,225] We have used size exclusion chromatography for exosome isolation, and analyses of our extracts with nanoparticle tracking, western blotting and electron microscopy demonstrate that this isolation method yields highly enriched vesicle populations with characteristics of exosomes. In line with recommendations from The International Society for Extracellular Vesicles,[26] we have provided detailed technical information on our collection and isolation methodologies to allow comparison with future studies of serum exosomes in MS and other disorders. Our results with and without RNaseA treatment are in line with previous studies indicating that exosomes provide a protective environment for RNA,[6] and that some miRNAs appear to be selectively packaged in exosomes[255].

In summary, this study demonstrates that exosomal-associated miRNAs have utility as biomarkers in MS. Our findings indicate that these biomarker profiles are distinct to those previously reported from serum or plasma circulating miRNA studies, while having comparable or superior predictive powers. Of note is the potential power to distinguish RRMS from progressive forms of the disease. The next generation of MS therapies offers the potential to specifically treat neuro-axonal and brain volume loss, and hence the ability to detect disease progression early may have major therapeutic and economic implications. If these exosomal biomarkers are able to indicate transformation to progressive disease earlier than current clinical methods, they are likely to have significant clinical utility. Longitudinal studies are needed to assess this question, and based on these initial investigations; these longitudinal studies should be pursued.

Chapter 3:

Characterising the effect of Fingolimod therapy on exosomal miRNAs in patients with relapsing-remitting MS

This chapter contains the original research article 'Characterizing the effect of Fingolimod therapy on exosomal miRNAs in patients with relapsing-remitting multiple sclerosis' which is in final preparation for submission to Neurology)

Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) and is a leading cause of neurologic disability in young adults. Although there is no definitive cure for MS, disease-modifying therapies have been shown to reduce disease progression by suppressing harmful inflammatory disease processes. Fingolimod is an orally administered immunomodulator, which prevents lymphocyte egress from lymph nodes and crosses the blood-brain barrier (BBB) having direct effects within the CNS. To manage treatment strategies and to tailor treatment to individual patients, there is an acute need to develop molecular biomarkers reflecting drug efficacy, and to determine drug-induced molecular processes to further the understanding of mechanisms of drug action and metabolism. Circulating exosomes carry distinct molecular cargo and represent promising candidate biomarkers for MS as they can be secreted from cells of the CNS and cross the BBB. It was hypothesised that the deregulation of serum exosomal microRNAs (miRNAs) expression is associated with the efficacy of Fingolimod therapy and is predictive of MS activity phases as determined by MRI, MS lesion activity. We profiled the expression of exosome-associated miRNAs in sera of 30 relapsing MS

patients prior to therapy and six months after treatment initiation using small RNA next generation sequencing. We then identified 15 miRNAs (i.e., miR-122-5p, miR-1246, miR-127-3p, miR-19b-3p, miR-134-5p, miR-323b-3p, miR-370-3p, miR-375, miR-379-5p, miR-382-5p, miR-411-5p, miR-432-5p, miR-485-5p, miR-493-3p, and miR-889-3p) differentially expressed in post-treatment active versus quiescent phases of MS, and determined the predictive power of individual and subsets of miRNAs using univariate and multivariate models. We also predicted biological processes potentially affected by dysregulated miRNA targets associated with MS disease activities using an external dataset of gene expression profiling of post-mortem MS lesions in active and quiescent phases of disease. Further, we identified miRNAs differentially expressed in drug responders associated with Fingolimod efficacy. Accordingly, we determined 11 miRNAs (i.e., miR-203a, miR-193a-5p, miR-379-5p, miR-370-3p, miR-382-5p, miR-493-3p, miR-432-5p, miR-485-5p, miR-2110, miR-1307-3p, miR-1908-5p) significantly dysregulated in stable responders whose disease phase remained inactive after treatment. We also identified 5 miRNAs (i.e., miR-150-5p and miR-548e-3p, miR-130b-3p, miR-654-5p, miR-487b-3p) differentially expressed in positive responders whose active MS phase turned to quiescent after six months of Fingolimod therapy. Pathways associated with dysregulated miRNAs were also predicted as potential molecular mechanisms induced in response to fingolimod treatment. As well as furthering the understanding of the action and metabolism of Fingolimod, this work also suggests that exosomal miRNA profiles have the potential to be utilised in MS clinical practice as biomarkers of disease activity and treatment response in the future.

Introduction

MS is a chronic inflammatory disease of the CNS characterised by demyelination and neurodegeneration.[256,257] MS affects approximately 2.5 million people worldwide.[256] Relapsing-remitting multiple sclerosis (RRMS) is the most prevalent subtype of the disease, occurred in about 85- 90% of patients.[127,258] Patients with RRMS experience an unexpected flare-up of symptoms (relapse) for days or weeks followed by substantial remission, often with some remaining disability.[127,259] Over time, as a result of disability accumulation and incomplete recovery following

relapses, up to 80% of RRMS patients develop secondary progressive MS with less inflammation and more neurodegenerative pathogenesis.[259,260]

There is no definitive cure for MS and no therapies are available to repair existing damage of CNS caused by MS.[10] Current disease modifying treatment (DMT) can help control the disease by suppressing the inflammatory response, slowing the progression of the disease and delaying lesion formation.[256,261,262] The list of Food and Drug Administration (FDA) approved drugs has been increasing from two in the 1990s to 12 in 2016.[127,262] Fingolimod (marketed as Gilenya®) is the first oral immunomodulatory medication approved for the treatment of RRMS in 2010.[263] It is a structural analog of sphingosine 1-phosphate (S1P) that acts as a S1P receptor modulator and prevents lymphocyte egress from lymph nodes. Fewer lymphocytes in the peripheral circulation reduces their infiltration into the CNS and limits myelin destruction.[238,263-265] Furthermore, Fingolimod is able to cross the BBB and have direct effects within the CNS by contributing to a reduction of the neurodegenerative processes and promoting repair mechanisms.[256]

To manage treatment strategies in individual MS patients, it is essential to study the contribution of patients' molecular profiles in determining drug efficacy and mechanisms of action.[266] Such pharmacogenomic approaches are aimed at developing genotype- or transcriptome-based predictive and markers of drug efficacy for improving and personalising drug therapy. Pharmacogenomics of miRNAs is an emerging field of research that holds promise for individualised tailor-made treatments.[267,268]

MiRNAs are small (18–25 nt), non-coding RNA, regulating gene expression post-transcriptionally.[3] A single miRNA can target multiple genes, while an individual mRNA may be regulated by distinct miRNAs.[4] This complexity reflects the involvement of miRNAs in the regulation of many molecular signalling pathways, such as in the immune system, and affecting various cellular processes, such as the function of immune cells.[5,6] Many drugs act by regulating specific genes. The expression level of these pharmacogenomic genes can be changed by regulatory functions of miRNAs. Thereby, miRNA expression can determine drug mechanisms and influence drug efficacy.[269]

Exosomes are membrane-bound vesicles released by almost all cell types; they carry a selective package of small regulatory RNA, such as miRNA, and can cross the BBB.[8,9] In our previous study, we have successfully profiled serum exosomal miRNAs to; (i) distinguish MS patients from health control (HCs), and (ii) separate the RRMS subtype from the progressive MS disease forms.[270] The effect of immunomodulatory therapy on the expression levels of miRNAs in MS patients has also been previously reported.[238,271,272]

In this study, we have used unbiased next-generation sequencing to consider miRNA deregulation within serum exosomes as markers of MS activity and therapeutic efficacy. We then used integrative bioinformatics to predict the functional role of dysregulated miRNAs. We hypothesised that the difference in serum exosomal miRNA profiles is predictive of disease activity in relapsing MS patients before and after Fingolimod therapy.

Gadolinium (Gd) enhancing MS lesions on MRI are indicative of early lesion development, active inflammation and breakdown of the BBB. MS patients without corresponding clinical changes can have Gd enhancing lesions on MRI. This clinical-radiological dissociation highlights why MRI is an important component of monitoring disease activity, and is a suitable tool for assessing treatment effectiveness.[273,274]

Overall, this is the first demonstration that miRNAs associated with circulating exosomes are informative, minimally invasive and cost-effective biomarkers of MS disease activity and treatment efficacy. The functional roles of dysregulated miRNAs were systematically and comprehensively investigated providing insights into the mechanisms of Fingolimod action in therapy responders.

Materials and methods

Experimental procedures

Study population

Relapsing MS patients naïve to Gilenya who attended the Royal Prince Alfred Hospital MS Clinic at the Brain and Mind Centre, The University of Sydney, Australia were enrolled in this study. Patients eligible for inclusion were diagnosed with MS according to McDonald 2010 criteria (as assessed retrospectively by analysis of clinical records and baseline MRI), have a relapsing disease course, disease duration <20 years, with expanded disability status scores (EDSS) 0-6.5 and normal kidney function. Patients were excluded if they had ever used cladribine, fludarabine, total body irradiation or alemtuzumab/Campath, received immunosuppressant agents in less than six months, or participated in any drug investigation trial or experimental procedure within the past 30 days. All patients assessed at baseline (prior to treatment) and six months with the same clinical and Gadolinium-based contrast agents for MRI scans (Gd-MRI). Patients were monitored with Gd-MRI, for relapses and rated using the EDSS scores. Written informed consent was obtained from each patient. This study was performed in accordance with relevant guidelines and regulations with the approval of the individual ethics committees of the institutions where the patients were being treated.

Patient data collection — clinical, MRI and blood

Prior to recruitment into this prospective study, written informed consent was obtained from each patient. Ethical approval for the study was through the University of Sydney Human Research Ethics Committee. All patients were assessed at baseline, prior to treatment with Fingolimod, and then at the six month mark, following Fingolimod commencement. Demographic and MS disease related data was documented at baseline and follow up for each patient and specifically included the collection of clinical relapse information and the performance of a formal EDSS score. Brain MRI with Gd was performed at both time points as well as a 12 month follow up using the same MRI protocol on the same 3T General Electric MRI

scanner located at the Brain and Mind Centre. The T1-weighted post-Gd MRI sequence was used in this study to assess for the presence or absence of MRI disease activity. Blood was collected from the patients at both time points and was stored (see section below). Clinical assessment, brain MRI with Gd and blood collection all took place at both baseline and six monthly follow up.

Exosome purification and characterisation

Blood samples were withdrawn from patients at baseline and six months at the time of Gd-MRI and clinical consultation. The serum sample (1 ml) was treated with RNaseA (37 °C for 10 minutes; 100 ng/ml; Qiagen, Australia) and accessed for exosome isolation by size exclusion chromatography (qEV iZON Science) as previously described.[270] Captured exosomes were characterised by nanoparticle tracking analysis, transmission electron microscopy and immunoblotting (outlined by the Society for Extracellular Vesicles[26]) to monitor particles concentration and size, morphology and the presence of exosomes membrane markers.

RNA extraction and small RNA sequencing

RNA isolation from serum exosomes was performed to construct small RNA sequencing libraries as previously described.[270] Briefly, purified exosomes were processed for RNA isolation using the Plasma/Serum Circulating & Exosomal RNA Purification Mini Kit (Norgen Biotek, Cat. 51000). The yield, quality, and size of extracted RNA were examined before constructing sequencing libraries from exosome RNA using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (BioLabs, New England). Finally, libraries were pooled with an equal proportion and sequenced on Illumina HiSeq.2000 System.

Statistical analyses

Data pre-processing and differential expression analysis

Data pre-processing was performed using a pipeline comprising of adapter trimming (cutadapt), followed by genome alignment to human genome hg 19 using Bowtie (18bp seed, 1 error in seed, quality score sum of mismatches <70). Where multiple

best strata alignments existed, tags were randomly assigned to one of those coordinates. Tags were annotated against mirBase 20 and filtered for at most one base error within the tag. Counts for each miRNA were tabulated and adjusted to counts per million miRNAs passing the mismatch filter. All samples achieved miRNA read counts >45,000 read counts and miRNAs with low abundance (<50 read counts across more than 20% of samples) were removed. Normalisation and differential expression analysis were performed using RNA-seq analysis tools in Bioconductor 'limma' package. Accordingly, read counts were first converted to log2-counts-per-millions to stabilize variances at high counts. The mean-variance relationship was then estimated at the individual observation level[275] to adjust for different count sizes across samples and combined with sample-specific quality weights to down-weight outlier samples[276] using 'voomWithQualityWeights' function. The transformed read counts were then entered the standard limma empirical Bayes method pipeline for differential expression analysis estimating moderated t-statistics and the corresponding *p*-values.[277] In any comparison, differentially expressed miRNAs were identified as those whose p-value < 0.05 with fold-change doubled in either direction (i.e., $|\log2 \text{ (fold-change)}| \geq 1$). The advantage of the above-mentioned limma strategy has been comprehensively confirmed in providing more powerful analysis and fewer false discoveries when compared to conventional approaches.[276]

Analysis of the predictive power of the identified miRNAs

Univariate logistic regression modeling

We performed logistic regression (LR) and receiver operator characteristic (ROC) analysis to assess the predictive power of individual miRNAs between the two groups of interest. LR was used to identify linear predictive models with each miRNA as the univariate predictor. The quality of each model was depicted by the corresponding ROC curve, which plots the true positive rate (i.e., sensitivity) against the false-positive rate (i.e., 1-specificity). ROC curves were smoothed using Tukey's method[278] to improve readability. The AUC was then computed as a measure of how well each LR model can distinguish between two comparative groups. The 90% confidence intervals (CI) of AUC measures were estimated using Delong method[279]

to assess the significance of a model's predictive power as compared to a random trial (i.e., AUC = 0.5). We then used leave-one-out cross validation (LOO-CV) to estimate the prediction errors of the LR models. LOO-CV learns the model on all samples except one and tests the learnt model on the left-out sample. The process is repeated for each sample and the error rate is the proportion of misclassified samples. These analyses were performed by R 'stats', 'boot' and 'pROC' packages using glm, cv.glm, roc and ci.auc functions, respectively.

Multivariate random forest modeling

The predictive power of multiple miRNAs multivariate signatures of MS activity was assessed using RF modeling. RF modeling is an ensemble learning method for classification/regression that operate by constructing a multitude of decision trees at training time in order to correct for the overfitting problem.[280] We used out-of-bag (OOB) error as an unbiased estimate of the test set prediction error as implemented by the R 'RandomForest' package.

Prediction of the functional role of the identified miRNAs

To predict the putative function of deregulated exosomal miRNAs in MS, we performed pathway and gene ontology enrichment analysis on targets of miRNAs of interest. We used MSigDB,[281] version 6.1, to retrieve KEGG pathways (186 pathways on 12,875 genes), Reactome pathways (674 pathways on 37,601 genes) and gene ontology (GO) biological processes (4436 GO terms on 506,182 genes). Human miRNA targets were retrieved from publicly available datasets of experimentally-validated and predicted datasets using *multiMiR*[282]— database v2.2, updated on 8/8/2017. MultiMiR is a miRNA-target interaction R package and database which compiles nearly 50 million records in human and mouse from 11 different databases: validated targets were collected from miRecords,[283] miRTarBase,[284] and TarBase [285] and predictions from DIANA-microT-CDS,[286] ElMMo,[287] MicroCosm,[288] miRanda,[289] miRDB,[290] PicTar, PITA,[291] and TargetScan[292]. Targets of miRNAs under study were included if experimentally-validated or predicted by at least two databases, and underwent pathway enrichment analysis using a one-sided Fisher's exact test, in which the *p*-value for the null hypothesis is computed based on the hypergeometric distribution:

$$p = \frac{1}{\binom{N}{n}} \sum_{i=k}^{i=n} \binom{n}{i} \binom{N-K}{n-i},$$

Where N is the total number of annotated genes in MSigDB, n is the number of genes targeted by miRNA(s), K is the total number of genes annotated by a pathway or gene ontology (GO) term, and k is the number of target genes annotated with a pathway or GO term. The nominal p-values were adjusted for multiple hypothesis tests using Benjamini & Hochberg (FDR) correction. Enrichment analyses were implemented in R using 'stats' packages.

Results

Patient characteristics and data preprocessing

Thirty relapsing MS patients were included in this study based on the selection criteria described in Materials and Methods. Patients were grouped based on their Gd-MRI scans to either active or quiescent before and after therapy. Samples from one patient were removed, as post-treatment MRI status was not available. Participant demographic and clinical characteristics are outlined in *Table 3-1*. The expression of serum exosomal miRNAs by deep sequencing were analysed at two distinct time points (i.e., baseline when the patients were treatment-naïve) and six months after patients started an immunomodulatory therapy with Fingolimod (Gilenya). The total of 1,924 miRNAs were screened for each sample; all samples achieved miRNA read counts >45,000. For each comparison, miRNAs with low abundance (<50 read counts across more than 20% of samples) were removed retaining around 11-12% of miRNAs in any comparison. Read counts were normalized to adjust for RNA and sample-level biases. There is no significant difference in age (using Mann–Whitney U non-parametric test) and gender (using Fisher's exact test) between any two groups compared in this study (p-value close to 1 in most of comparisons).

Table 3-1 Characterisation of patients

Characteristic	n = 29	Description
Gender (F/M)	17/12	F: Female, M: Male
Age	38.8 ±10.1	Average age at enrolment, mean ± std
Age of onset	32.9 ±10	Age when MS was first diagnosed, mean ± std
Disease duration	61.3 ± 80 month	Duration of disease from the first diagnosis, mean ± std
Active (pre/post)	14/8	Number of patients in active or quiescent phases of MS (based on Gd-MRI) before & after therapy
Quiescent (pre/post)	15/21	
Therapy	Fingolimod (Gilenya)	Immunomodulatory medication

Deregulation and function of serum exosomal miRNAs in MS activity

Exosomal miRNAs are potential markers of MS activity phases

At each timepoint, we compared miRNA profiles of patients in active vs quiescent phases as determined by Gd-MRI scans (*cf.* (*Figure 3-3 –A* and *Table supplementary A-1* for the list of patients in each comparison and their clinical characteristics). In the baseline, miR-194-5p and miR-374a-5p were differentially expressed based on the adopted criteria (i.e., |log2 (fold change)|>1 and *p*-value <0.05 using limma linear model and empirical Bayes method for assessing differential expression), both up regulated in the active compared to the quiescent phase. Six months after the Fingolimod treatment, the number of significantly deregulated miRNAs increased to 15 including miR-122-5p, miR-1246, miR-127-3p, miR-19b-3p, miR-134-5p, miR-323b-3p, miR-370-3p, miR-375, miR-379-5p, miR-382-5p, miR-411-5p, miR-432-5p, miR-485-5p, miR-493-3p, and miR-889-3p, where all, except miR-q9b-3p, were up-regulated in the active phase (*Figure 3-1A)*. The increase in the number of post-treatment differentially expressed miRNAs in MS patients is consistent with the previous report on the study of patients' RNA deregulation in response to the interferon ß treatment,[272] and potentially reflects an improved within-group homogeneity ensued from the administration of the medication. To estimate within-group sample heterogeneity, we measured variance of each miRNA expression

values across samples within each active and quiescent group, before and after treatment. The mean of variances significantly reduced in quiescent as well as active groups after-treatment compared to pre-treatment (*p*-value = 1.571e-05 comparing quiescent groups and *p*-value = 0.0207 comparing active groups after vs before therapy, using Mann–Whitney U non-parametric test). This can imply that reduced after-therapy heteroscedasticity ensued improved statistical hypothesis testing (reducing type II error). Hence, subsequent analyses were focused on 15 miRNAs dysregulated in after treatment MS activity phases.

Next, we examined the predictive power of each dysregulated miRNA using a univariate LR model whose predictor was the individual miRNA expression profile. ROC curves were determined for each candidate miRNA, where the true positive rate, sensitivity, is plotted against the false positive rate and i.e., 1 – specificity *(Figure 3-1B).* Area under the ROC curve (AUC) measures and the corresponding 95% confidence interval estimates were then computed for each miRNA *(Figure 3-1B).* Only miRNAs whose AUC confidence intervals do not contain the null hypothesis value (AUC = 0.5 for a random prediction) were considered as statistically accurate univariate predictors of MS activity phases *(Figure 3-1B).* Accordingly, 11 miRNAs dysregulated in post-treatment samples were selected (i.e., miR-1246, miR-127-3p, miR-19b-3p, miR-134-5p, miR-370-3p, miR-375, miR-379-5p, miR-382-5p, miR-432-5p, miR-485-5p, and miR-493-3p) and used as predictors of a linear multivariate LR model as well as a random forest (RF) model that is a nonlinear classifier, to investigate whether the combined expression patterns of multiple miRNAs could improve the predictive power. RF model achieved a higher predictive power compared to LR (i.e., the prediction error rates were 0.39 and 0.35 in LR and RF, respectively). We therefore, opted for the RF model for subsequent multivariate analyses. We were interested to identify an *optimal* miRNA signature that is to find a minimum set of miRNAs whose combined expression patterns predict MS phases with the highest accuracy. Accordingly, all possible combinations of 11 miRNAs were trialled (the total of 2,037 signatures comprising 2 to 11 miRNAs); the corresponding RF multivariate models were then generated, and OOB error rates estimated. A combination of two or three miRNAs provided a predictive power of 92% for distinguishing active from quiescent RRMS phases *(Figure 3-1C).*

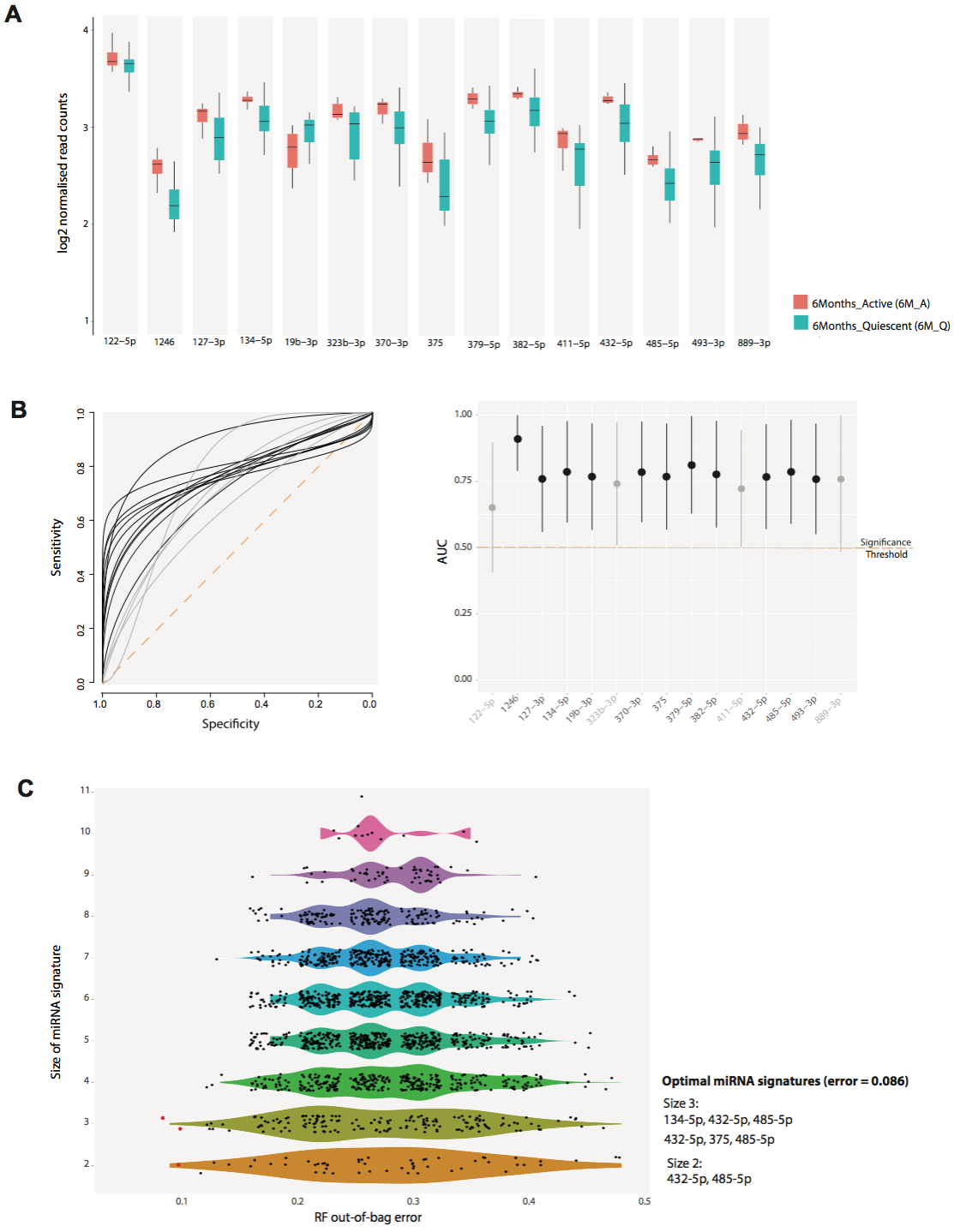


Figure 3-1 Exosomal miRNAs as markers of disease activity

A) Differentially expressed miRNAs were identified by limma linear model and empirical Bayes method with |log2 (fold change)|>1 and *p*-value < 0.05. Box-and-whisker plot represents for each miRNA species between active and quiescent patients 6 months after Fingolimod treatment. B) To examine the predictive power of each dysregulated miRNAs ROC analysis performed on individual miRNAs. AUC measured and the corresponding 95% confidence interval estimates were then

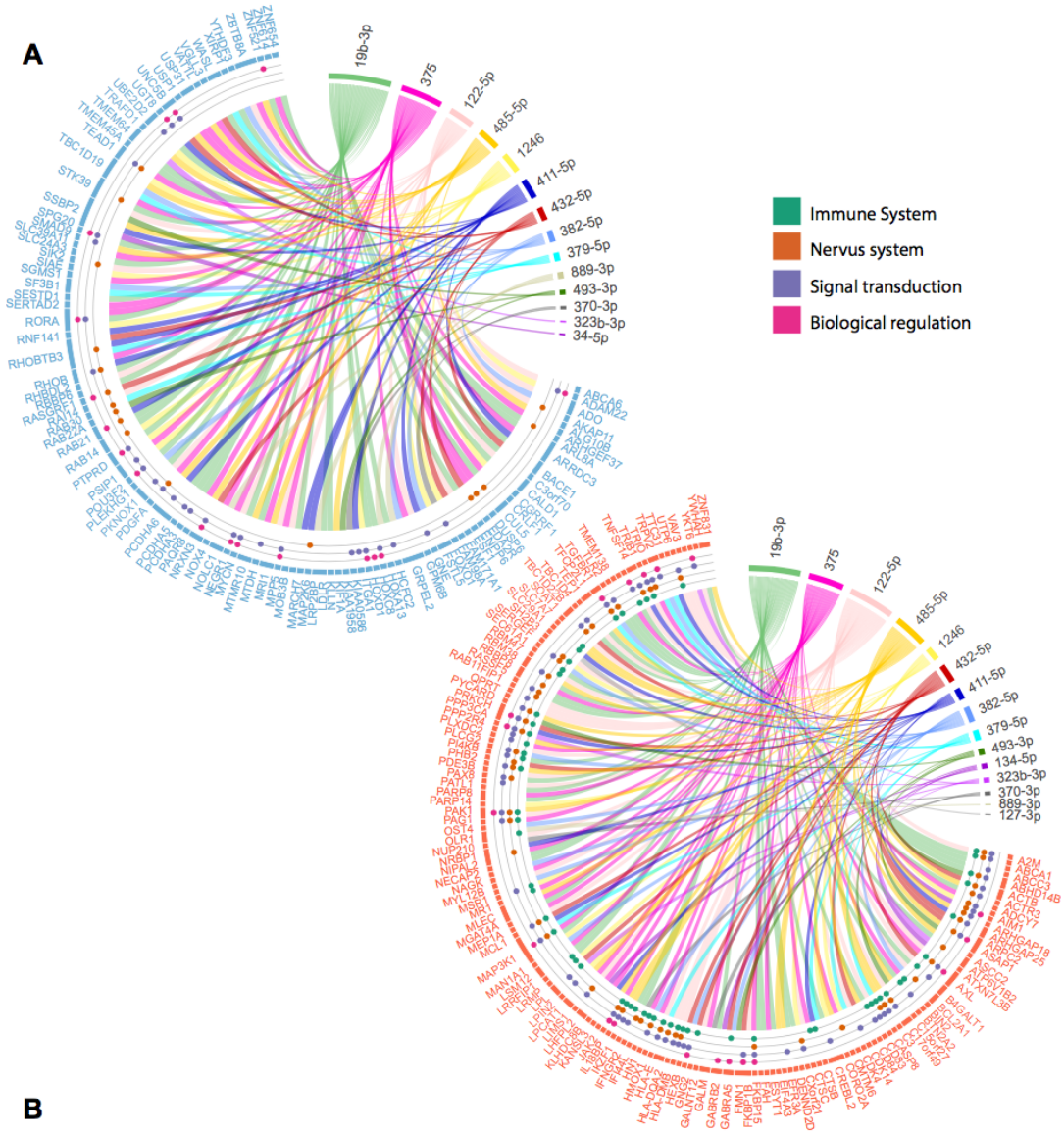
computed for each miRNA. 11 miRNAs with AUC confidence more than 0.5 were considered as statistically accurate univariate predictors of MS activity phases. C) RF model was run using all possible combinations of 11 dysregulated miRNAs to improve the predictive power. The corresponding RF multivariate models were then generated, and OOB error rates estimated. A combination of two or three miRNAs provided a predictive power of 92% for disease activity.

Prediction of the functional role of dysregulated miRNAs

To predict the potential function of miRNAs dysregulated in active *vs* quiescent RRMS phases, we first retrieved gene targets of differentially expressed miRNAs from multiple miRNA-target interaction databases and selected those targets that are either experimentally validated or computationally predicted in at least two datasets. Accordingly, 4,650 targets were retrieved for 15 miRNAs dysregulated in post-treatment active *vs* quiescent samples.

To enhance the specificity of subsequent functional analysis, we sought to identify targets specific to disease activity in MS brain lesions. Hence, we used a previously generated gene expression profiling of post-mortem brain tissues of MS patients in active and inactive RRMS phases.[293] We identified genes differentially expressed in chronic active *vs* inactive MS lesions (RIM)—i.e., p-value < 0.05 using limma microarray linear model fit and empirical Bayes method on normalized gene expression data retrieved from GSE108000.

We identified 153 and 102 target genes to be upregulated and downregulated in active compared to the inactive lesions, respectively. These targets were then undergone enrichment analysis for gene ontology biological processes. Overrepresented biological processes (FDR <0.01) were summarised and stratified under four categories of immune system, nervous system, signal transduction and biological regulation by consulting gene ontology hierarchy. *Figure 3-2A* visualises the network of dysregulated miRNAs interacting with the target genes up/down regulated in MS lesions. Targets were annotated with biological processes categories if the gene has been annotated by at least one GO term within the associated category *(Figure 3-2B).* Similar enrichment analyses were performed on curated pathways (KEGG and Reactome).



Immune Sy.

Biological processes enriched by up-regulated genes: Immune system process(7.6E-23), Regulation of immune system process(1.0E-19), Regulation of innate immune response (1.5E-07), Defense response(2.0E-07), Regualation of cytokine production(3.6E-07), Phagocytosis(8.8E-07), Response to cytokine (9.3E-06), leukocyte activation(1.4E-03), Regulation of immune effector process(1.8E-03), lymphocyre activation(2.8E-03), Regulation of type I interferon production (3.6E-03), Immune system developmnet(4.1E-03), Regulation of chemokine production(5.4E-03), Cellular response to cytokine stimulus(6.0E-03), Cellular response to interferon-gamma(6.3E03), Positive regulation of interleukin 1-beta production(7.0E-03),Positive regulation of macrophage dervied foam cell differentiation(3.6E-04), Regulation of leukocyte proliferation(9.7E-03), Regulation of inflammatory response(9.4E-03),Wound healing(1.4E-10)

Nervous Sy.

Biological processes enriched by up-regulated genes: Neurogensis (2.2E-03) and negative regulation of dendrite development (5.3E-03)

Biological processes enriched by down-regulated genes: Neurogenesis (3.0E-07), Neuron differentiation (3.0E-04), Peripheral nervous system develpment (8.2E-04), Ensheathment of neurons (3.3E-03), Peripheral neruvous system axon ensheathment (3.3E-04), Schwann cell development (6.7E-04) and Schwann cell differentiation (1.4E-03)

Signal Transduc.

Biological processes enriched by up-regulated genes: Intracellular signal transduction (3.4E-13), Regulation of intracellular signal transduction (4.6E-09), Regulation of GTPase activity (3.7E-05), FC receptor signaling pathway (3.7E-05), Regulation of protein activation cascade (2.3E-04), Interferony mediated signaling pathway (2.3E-04), Enzyme linked receptor protein signaling pathway (4.0E-04), Ephrin receptor signaling pathway (7.4E-04), Transmemebrane receptor protein trosine kinase signaling pathwat (8.8E-04), Positive regulation of intracellular signal transduction (9.5E-04), Antigen receptor mediated signaling pathway (6.7E-03), Regulation of small GTPase mediatated signal.

Biological processes enriched by down-regulated genes: Intracellular signal transduction (1.8E-06), Regulation of intracellular signal transduction (3.09E-05) and small GTPase mediated signal transduction (8.08E-05)

Biological Reg.

Biological processes enriched by up-regulated genes: Regulation of cell differenctiation (4.3E-10), Positive regulation of gene expression (3.0E-06), Regulation of cell proliferation(6.7E-06), Regulation of cell morphogenesis involved in differentiation(2.5E-03), Negative regulation of cell communication(6.9E-03), Regulation of sequence specific DNA binding transcription factor activity(4.5E-04), Positive regulation of cell development (5.1E-04), Positive regulation of actin filament polymerization(7.2E-03), Regulation of cellular component movement (9.6E-03) and regulation of cell activation (2.2E-10)

Biological processes enriched by down-regulated genes: Regulation of transcription from RNA polymerase II promoter (3.4E-03), Biological adhesion (2.2E-04), Anatomical structure formation involved in morphogenesis(6.2E-03) and cell-cell adhision (7.9E-03).

Figure 3-2 Predicted functional role of miRNAs associated with MS disease activity

A) Target genes of 15 miRNAs differentially expressed in active vs quiescent phases of MS were retrieved. Target specific to disease activity in MS brain lesions were then identified using GSE108000. Accordingly, 153 and 102 target genes were identified to be upregulated (red) and downregulated (blue) which annotated with biological processes categories; immune system, nervous system, signal transduction and biological regulation. B) A summary list of overrepresented GO terms.

Deregulation and function of exosomal miRNAs in treatment responders

Exosomal miRNA deregulation is associated with Fingolimod efficacy

To investigate miRNAs pharmacogenomic roles in response to Fingolimod therapy, we monitored MS activity phases of patients based on Gd-MRI scan results prior to therapy (baseline) and six months after treatment commencement and profiled their RNA-seq expression of exosomal miRNAs at each time point. Fifteen patients were indicated the quiescent phase at the baseline out of which 12 were remained in quiescent phase after six months *(Figure 3-3).* Also, out of 14 patients indicating active MS phase initially, nine were turned to quiescent mode after six months of Fingolimod treatment *(Figure 3-3).* Accordingly, the two main groups of **stable responders** (i.e., patients who were quiescent at baseline and six months after treatment) and **positive responders** (i.e., patients who were active at baseline and become quiescent after six months of treatment) were considered for differential expression analysis of exosomal miRNAs. Responders were stratified into the two groups to improve within-group homogeneity and reduce false negative rate (type II error). In the stable responder group, 11 miRNAs were dysregulated based on the adopted criteria (i.e., |log2 (fold change)|>1 and p-value < 0.05) including miR-203a, miR-193a-5p, miR-379-5p, miR-370-3p, miR-382-5p, miR-493-3p, miR-432-5p, miR-485-5p, miR-2110, miR-1307-3p, miR-1908-5p, all upregulated at six months after treatment *(Figure 3-3).* In the positive responder group, the expression level of miR-150-5p and miR-548e-3p decreased, while the level of the expression of miR-130b-3p, miR-654-5p, miR-487b-3p increased after treatment *(Figure 3-3).*

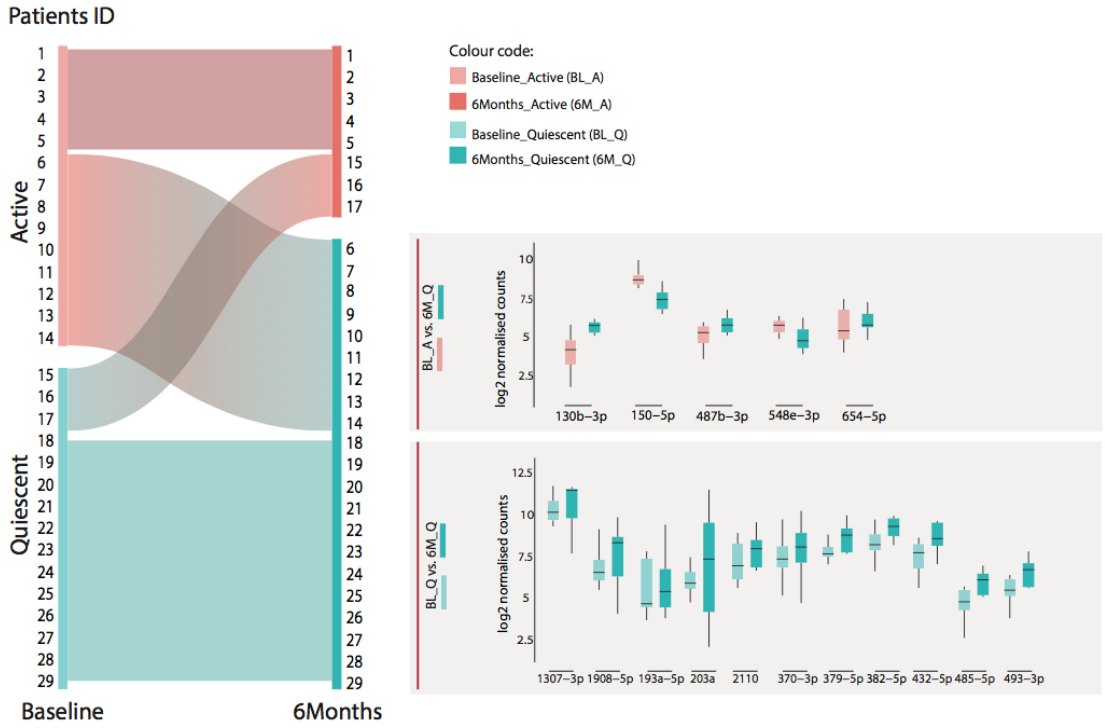


Figure 3-3 miRNAs deregulation associated with drug response

MS disease activity phase, based on Gd-MRI scan results, monitored at baseline (15 quiescent and 14 active), and six months after commencement of Fingolimod treatment. Twelve out of 15 quiescent patients were remained in (stable responders) and nine out of 14 active patients were turned (positive responder) to quiescent phase after six months treatment. Then differentially expressed miRNAs were identified based on the adopted criteria |log2 (fold change)|>1 and p-value < 0.05 which represents 11 and 5 dysregulated miRNA in stable and positive responder groups as shown in the box-and-whisker plots.

All patients remained on therapy for a longer period of time and monitored by Gd-MRI scan at 12 months after treatment initiation *(Table supplementary A-1)*. Seventy two per cent of patients were therapy responders at six-month post-treatment. This proportion increased to 90% in 12 months of treatment. Overall, the effect of Fingolimod therapy was adequately evident six months after treatment and was persistent for responder patients (except for patient 13 who indicated active MS in 12-month post-therapy). On the other hand, patients not responded to the treatment in the six-month follow-up, have generally shown improved responses by 12 months of

treatment (five out of eight non-responders at six-month have turned to quiescent phase of MS at 12-month follow-up as indicated by Gd-MRI results). Accordingly, while expanding the course of medication improves the outcome, six months of therapy sufficiently reflects the Fingolimod effect and miRNA regulatory changes associated with the treatment efficacy. This observation is further corroborated by a former study on the effect of Fingolimod treatment on circulating miRNAs indicating that miRNA expression profile significantly changes after six months of therapy.[238]

Prediction of the functional role of dysregulated miRNAs

We were interested to investigate pathways potentially perturbed by dysregulated miRNAs to further understand how the identified pharmacogenomic miRNAs are affecting mechanisms of Fingolimod action and metabolism. Accordingly, for each differentially expressed miRNA associated with drug efficacy, we retrieved target genes from 16 miRNA-target interaction databases and selected those targets that are either experimentally validated or computationally predicted in at least two datasets *(Figure 3-4A)*. We then performed pathway enrichment analysis using KEGG[294] and Reactome[295] databases comprising 860 pathways in total, to identify pathways overrepresented by targets of each miRNA *(Figure 3-4B)*. Pathways enriched by multiple miRNAs propose more robust association with drug-induced perturbation. We therefore sorted pathways by total number of associated miRNAs and chose the top 10% of pathways (i.e., pathways enriched by ≥ 5 miRNAs) as shown in *Figure 3-4C*. Selected pathways were sorted under 6 general categories of **immunity system**, **nervous system**, **signal transduction**, **lipid metabolism**, **diseases**, and **cell cycle** by consulting Reactome and KEGG pathway hierarchies (*Figure 3-4D)*. Multiple pathways relevant to the pathophysiology of MS and therapeutic targets are frequently enriched by miRNAs dysregulated in response to therapy offering alternate mechanisms of Fingolimod actions (*c.f.* discussion section for examples). Besides, *miR-130b-3p*, *miR-150-5p*, *miR-2110*, directly target S1P enriching known Fingolimod-induced pathways such as **sphingolipid metabolism**[296] and **sphingolipid de novo biosynthesis**[256].

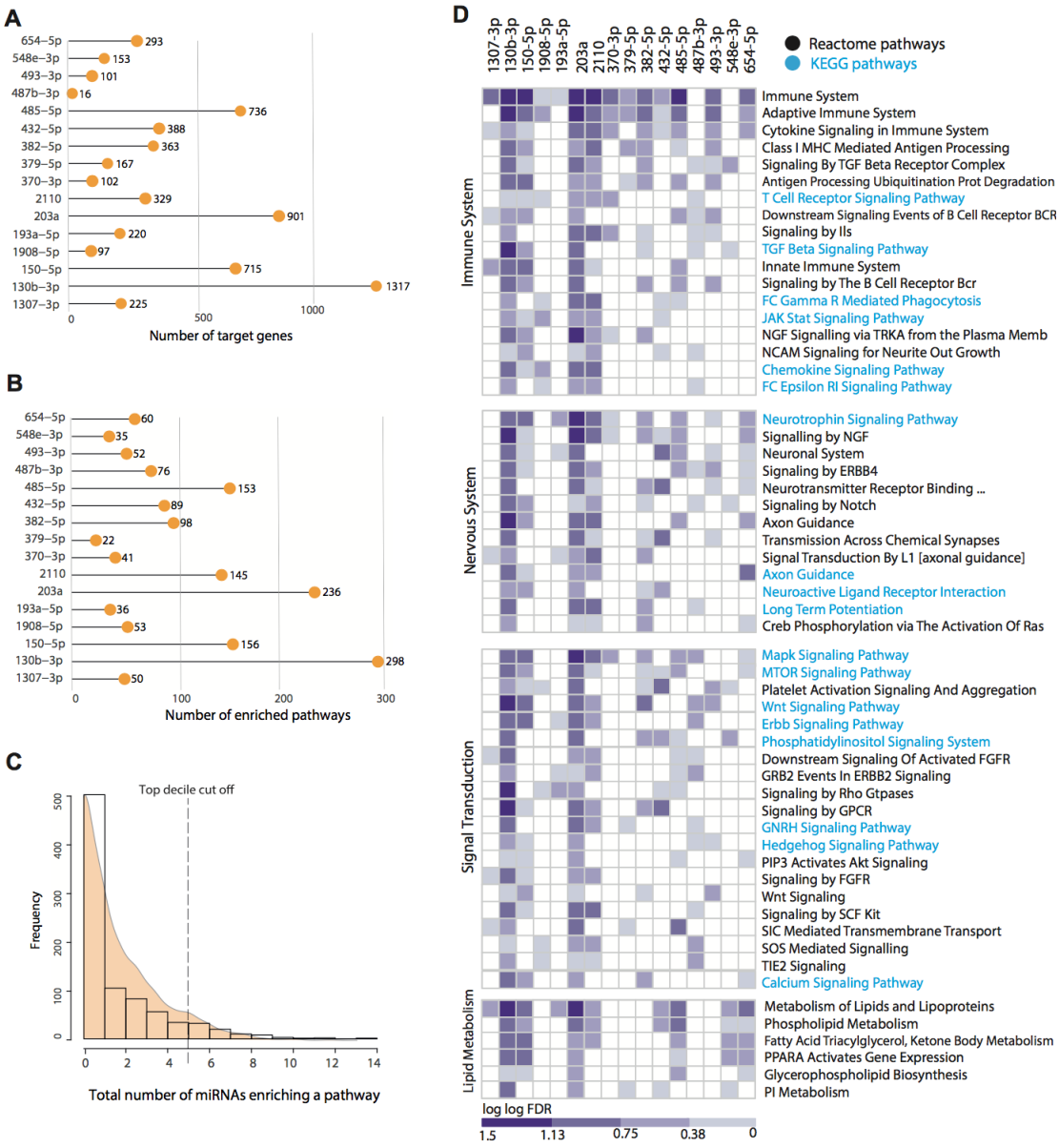


Figure 3-4 Functional roles of dysregulated pharmacogenomic miRNAs

A) The total number of target genes for each of 16 dysregulated miRNAs. B) Pathway enrichment analysis performed to identify overrepresented pathways by targets of each identified pharmacogenomic miRNA. The number of enriched pathways by targets of each dysregulated miRNA displayed. C) Pathways enriched by multiple miRNAs propose more robust association with drug-induced perturbation. This bar chart shows the distribution of a total number of miRNAs enriching a pathway across all pathways. The top 10% of robust pathways enriched by at least five miRNAs chosen. D) Selected pathways (top decile cut off) classified into four categories by consulting Reactome (black) and KEGG (blue) hierarchies.

Discussion

There is a growing body of evidence highlighting the regulatory role of miRNAs in the pathogenesis of MS and therapeutic impacts.[170,174,238,270,271,297-303] In this study, we have used unbiased high-throughput sequencing on serum exosome miRNAs capturing the complete profile of miRNAs in patient sera. We used size exclusion chromatography for exosome isolation—a method that is known for high reproducibility and purity of exosome extracts[69]—coupled with RNAse treatment of extracts in order to interrogate exosomal-associated miRNAs as a source of biomarkers distinct from free circulating miRNAs[270].

We identified miRNAs differentially expressed in patients experiencing relapse compared to remitting MS phases, as determined by Gd-MRI scans results. Machine-learning approaches on dysregulated miRNAs were used to examine their individual and collective predictive powers in discriminating disease phases. Eleven differentially expressed miRNAs (miR-1246, miR-127-3p, miR-19b-3p, miR-134-5p, miR-370-3p, miR-375, miR-379-5p, miR-382-5p, miR-432-5p, miR-485-5p, and miR-493-3p) have shown significantly high predictive power using logistic regression univariate analyses which subsequently used as predictors of random forest multivariate models. A combination of just two (miR-432-5p, miR-485-5p) or three miRNAs (miR-134-5p, miR-432-5p, miR-485-5p as well as miR-432-5p, miR-375, miR-485-5p) had a 92% accuracy rate of predicting active from quiescent RRMS phases as identified by random forest analyses.

Among the identified miRNAs, some have been previously indicated as circulating markers in MS or other immune system or nervous system related diseases. Yet, the majority of candidate miRNAs we have identified are novel, which likely reflects the unique constituent profile of exosomes versus free circulating miRNAs. Circulating miR-375 was shown to be dysregulated in sera of PPMS patients compared to controls.[304] The upregulation of miR-1246 has been previously reported in an active phase of systemic lupus erythematosus, a severe autoimmune disease, compared to the disease inactive phase.[305] Also, deregulation of miR-1246 in both naïve and regulatory T cells has been previously reported and identified as a marker characterizing the regulatory T cells phenotype.[306] They have demonstrated that miR-

1246 upregulation leads to the development of systemic autoimmune-like conditions in mice.[306] MiR-19b-3p, downregulated in active vs quiescent post-treatment RRMS patients in our study, has been previously reported to be under-expressed in serum of Alzheimer's disease patients compared to HCs.[307]

Further, we developed an integrative bioinformatics pipeline to investigate the functional role of dysregulated miRNAs by constructing a comprehensive yet reliable network of miRNA-target gene interactions and improving prediction specificity by considering target genes associated with MS activity in brain lesions. Multiple biological processes relevant to MS activity were enriched by target genes under four categories of immune system, nervous system, signal transduction and biological regulation. The importance of immune system genes in the pathogenesis of MS has been frequently reported.[308] Within the overrepresented biological processes, we found canonical immune-associated pathways such as **positive regulation of interleukin 1 Beta production**, which plays a role in MS-associated neurodegenerative damage and clinical progression.[309] Other two outstanding immune-associated pathways are **leukocytes activation** and **regulation of type I interferon production**. In a healthy CNS, leukocytes have limited access to the brain and spinal cord, whereas in several neurological diseases, including MS, leukocytes infiltrate from the periphery into the CNS resulting inflammation.[310] Conversely, type 1 interferon is an immunomodulatory cytokine with anti-inflammatory effect by controlling interleukin I.[160] Overall, our functional predictions strongly support the role of dysregulated miRNAs in MS activity, corroborating the validity of novel exosomal miRNA biomarkers and providing further insights into disease pathogenesis.

Investigating the role of miRNAs in the pathogenesis of MS and identifying miRNA-based pharmacogenomic markers of the treatment response is an active field of research.[160,271,297-299] This study was primarily aimed to interrogate the efficacy of Fingolimod therapy on RRMS patients in a prospective study monitoring disease progression based on Gd-MRI scan results and to assess the effect of treatment on patients' circulating exosomal miRNA profiles. We identified the total of 16 differentially expressed miRNAs associated with Fingolimod treatment efficacy. These include exosomal miRNAs dysregulated in sera of patients whose disease

phase remained inactive (i.e., miR-203a, miR-193a-5p, miR-379-5p, miR-370-3p, miR-382-5p, miR-493-3p, miR-432-5p, miR-485-5p, miR-2110, miR-1307-3p, miR-1908-5p) or turned to inactive phase (i.e., miR-150-5p, miR-548e-3p, miR-130b-3p, miR-654-5p, miR-487b-3p) after six months of treatment. Deregulation and function of some of predicted miRNAs have been previously reported in peripheral blood of MS patients. The altered expressions of miR-130b and miR-203 have been previously reported in B-cells from peripheral blood samples of RRMS patients.[160] These two miRNAs in our study target the highest number of genes over representing multiple pathways involved in immune system, nervous system, lipid metabolism as well as critical signal transduction. The same study also reported the deregulation of miR-150 in B-cell of blood samples after treatment of MS patients with Natalizumab.[160] The altered expression of miR-150 is also reported in T-cell[311] and peripheral blood mononuclear cells of MS patients compared to HCs[312]. The downregulation of miR-193a-5p has been previously reported in peripheral blood mononuclear cells of RRMS patients after six months Interferon-beta therapy.[313]

We also identified pathways consistently overrepresented by targets of multiple miRNAs associated with treatment response. Several pathways pivotal in MS pathogenesis and relevant to Fingolimod mechanisms were enriched and stratified under categories immune system, nervous system, signal transduction and lipid metabolism. Notably, T cells development and function is a common sight of disease pathogenesis and treatment response.[313] transforming growth factor beta signalling pathway regulates differentiation of naïve CD4 T-cell into regulatory T cell and reduction in this signalling pathway results in less number of regulatory T cells observing in MS patients.[314,315] Our results indicate that upregulated miRNAs in response to Fingolimod perturb Transforming growth factor beta signalling pathway, which in turn may ensue reduced susceptibility to developing MS. Another predicted pathway JAK-STAT pathway, which has indirect effect on interleukin-7 expression, an important cytokine for the regulation of B-cell and T-cell development and overexpressed in brain lesions of MS patients.[314] Additionally, the role of JAK-STAT pathway in interleukin-12 regulation has been reported to induce the expression of interleukin-7 mRNA and protein in microglia, macrophages and astrocytes.[314] Another interesting pathway is Wnt signalling pathway enriched by

targets of seven pharmacogenomic miRNAs in our study. Wnt pathway modulates the immune response and involves in the process of remyelination by controlling the balance between immune tolerance/inflammation and neuronal survival/neurodegeneration in MS.[316]

In summary, our results demonstrate that exosomal miRNAs are involved in MS immuno-pathogenesis and molecular mechanism of Fingolimod. We identified miRNAs perturbed in serum exosomes of treatment responders and predicted their impact on a variety of pivotal regulatory pathways. Nevertheless, due to the efficacy of therapy, the number of non-responder patients was too small to perform statistical comparison developing predictive markers of treatment positive vs negative response. To assess this question, longitudinal studies on a large cohort of RRMS patients are needed and based on these initial investigations; these longitudinal studies should be pursued.

Chapter 4:
Deep sequencing of circulating exosomal microRNA allows non-invasive glioblastoma diagnosis

This chapter contains the original research article 'Deep sequencing of circulating exosomal microRNA allows non-invasive glioblastoma diagnosis' which is under review in the npj Precision Oncology (2018).

Abstract

Exosomes are nano-sized extracellular vesicles released by many cells that contain molecules characteristic of their cell-of-origin, including microRNA (miRNA). Exosomes released by glioblastoma cross the blood-brain-barrier (BBB) into the peripheral circulation, and carry molecular cargo distinct to that of 'free-circulating' miRNA. Serum exosomal-microRNAs were isolated from glioblastoma (*n*=12) patients and analysed using unbiased deep sequencing. Results were compared to sera from age- and gender-matched healthy controls (HCs), and to grades II-III (*n*=10) glioma patients. Significant differentially expressed microRNAs were identified, and the predictive power of individual and subsets of microRNAs were tested using univariate and multivariate analyses. Additional sera from glioblastoma patients (*n*=4) and independent sets of healthy (*n*=9) and non-glioma (*n*=10) controls were used to further test the specificity and predictive power of this unique exosomal-microRNA signature. Twenty-six microRNAs were significantly differentially expressed in serum exosomes from glioblastoma patients' relative to HCs. Random forest (RF) modelling and data partitioning selected seven miRNAs (miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p and miR-543) as the most stable for classifying glioblastoma. Strikingly, within this model, two iterations of these miRNA classifiers could distinguish glioblastoma

patients from controls with perfect accuracy. The seven-miRNA panel was able to correctly classify all specimens in validation cohorts (*n*=23). Also identified were 23 dysregulated miRNAs in mutant isocitrate dehydrogenase isoforms (IDH^{MUT}) gliomas, a partially overlapping yet distinct signature of lower grade glioma. Serum exosomal-miRNA signatures can accurately diagnose glioblastoma preoperatively. miRNA signatures identified are distinct from previously reported 'free-circulating' miRNA studies in glioblastoma multiforme (GBM) patients, and appear to be superior.

Introduction

Malignant gliomas, particularly GBM, represent the most lethal primary brain tumours, owing in part to their highly infiltrative growth patterns. The World Health Organization's guidelines sub-categorise glioma by histopathologic evaluation into tumour grades I-IV, where GBM (grade IV) is the most aggressive and also the most common. Despite surgery, radiation, and chemotherapy, essentially all GBM tumours recur, at which point patients have reduced treatment options and worsening prognoses. Compounding this aggressive cancer phenotype are challenges in monitoring responses to treatment and tumour progression. While recent revisions to the Response Assessment in Neuro-oncology criteria helps to standardise glioma tumour monitoring,[317] radiographic measurements can be unreliable and insensitive to early signs of treatment failure and tumour relapse. Moreover, there are still difficulties deciphering pseudo-progression and pseudo-responses in some patients. Brain biopsy and histologic analysis can provide definitive diagnoses and evaluation of disease progression, however serial biopsies are impractical given the cumulative surgical risk, and biopsied tissue may not reflect the heterogeneity of GBM tumours.

An important step towards the provision of personalised GBM patient care is the ability to assess tumours *in-situ*. As such, there is a real need for biomarkers that can measure disease burden and treatment responses in GBM patients in a safe, accurate and timely manner, and preferably before changes become clinically apparent. The recently popularised idea of 'liquid biopsy' presents an ideal approach to monitor GBM tumour load and evolution in response to treatment. If developed and

implemented alongside new treatments, such tests would provide useful surrogate endpoints and allow clinical trial protocols to be more dynamic and adaptive.

Exosomes are nano-sized (30–100 nm) membrane-bound extracellular vesicles released by all cells in both health and disease, and there is growing interest in their use as non-invasive biomarkers for disease diagnosis and monitoring of disease recurrence.[318] GBM-derived exosomes circulate in the peripheral blood of patients, and can contain diagnostic nucleic acid.[41] We recently described a GBM exosome protein signature[319] and also showed that GBM exosomes contain abundant, selectively packaged sncRNAs.[255] Using unbiased sncRNA deep sequencing, we identified several unusual and/or completely novel sncRNAs within GBM exosomes *in vitro* as well as an enrichment of miRNA implicated in oncogenesis, including miR-23a, miR-30a, miR-221 and miR-451.[255] Thus, while GBM exosomal miRNA contents broadly reflect their cell of origin, there is a unique profile of miRNAs within exosomes.

Some studies of exosomal miRNA in GBM patients have already been reported; these studies utilised methods that focused on pre-defined and relatively small groups of miRNA species. One previous study found that miR-21 levels in CSF exosomes of GBM patients were up-regulated 10-fold compared to controls,[320] while another reported that serum exosomal miR-320, mir-547-3p, and RNU6-1 were significantly associated with GBM diagnosis, as well as outcome (RNU6-1)[221]. However, to date no comprehensive analysis of the entire miRNA repertoire of serum exosomes in glioma patients has been performed. Here, we have used unbiased next generation sequencing and an integrative bioinformatics pipeline[270] to assay the complete repertoire of exosomal-associated miRNAs in the serum of patients with glioblastoma, lower grade gliomas, and HCs. We describe a novel miRNA signature within serum exosomes that is highly predictive of pre-operative GBM diagnosis. Furthermore, we show that this approach has potential for describing unique miRNA signatures for distinct glioma entities.

Material and methods

Participants

Serum (1 ml) was accessed from the Neuropathology Tumour and Tissue Bank at Royal Prince Alfred Hospital (RPAH), New South Wales, Australia (Sydney Local Health District, Human Research Ethic Committees (HREC) approval, X014-0126 & HREC/09RPAH/627). Twenty-six serum specimens were collected pre-operatively from patients with histologically confirmed glioma tumours, including 16 with GBM, IDH-wildtype (IDH^{WT}) WHO (2016) grade IV, and 10 patients with grade II-III IDH-mutant (IDH^{MUT}) gliomas (refer to *Table 4-1* and *Table 4-2*). Age- and gender-matched healthy control sera (*n*=16) were used for discovery miRNA analyses. Sera from an additional nine HCs and ten non-glioma patients (active multiple sclerosis, *n*=9, and ganglioglioma, *n*=1) were used to test the GBM miRNA signature. This study was performed under RPAH, and The University of Sydney HREC approved protocols (#X13-0264 and 2012/1684), and all participants provided written informed consent. All methods were performed in accordance with the relevant guidelines and regulations.

Exosome purification and characterisation

Exosomes were isolated from serum as previously described.[270] Briefly, serum (1 ml from each subject) was treated with RNase A (37 °C for 10 min; 100 ng/ml; Qiagen, Australia) before exosome purification by size exclusion chromatography (qEV iZON Science). Ten fractions (500 µl) were eluted in phosphate buffered saline (PBS), as per manufacturer's instructions. Fractions 8, 9, and 10 were previously shown to contain purified exosome populations[270] and were collected and stored at −80 °C. Captured exosomes were characterised in accordance with the criteria outlined by the International Society for Extracellular Vesicles.[26] Specifically, we identified more than three exosome-enriched proteins by mass spectrometry proteome profiling and characterised vesicle heterogeneity using two technologies, transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA).

Transmission electron microscopy

Combined qEV-captured fractions 8-10 was loaded onto carbon-coated, 200 mesh Cu formvar grids (#GSCU200C; ProSciTech Pty Ltd, QLD, Australia), fixed (2.5% glutaraldehyde, 0.1 M phosphate buffer, pH7.4), negatively stained with 2% uranyl acetate for two minutes and dried overnight. Exosomes were visualised at 40,000 times magnification on a Philips CM10 Biofilter TEM (FEI Company, OR, USA) equipped with an AMT camera system (Advanced Microscopy Techniques, Corp., MA, USA) at an acceleration voltage of 80 kV.

Nanoparticle tracking analysis

Particle size distributions and concentrations were measured by NTA software (version 3.0) using the NanoSight LM10-HS (NanoSight Ltd, Amesbury, UK), configured with a 532-nm laser and a digital camera (SCMOS Trigger Camera). Video recordings (60 s) were captured in triplicate at 25 frames with default minimal expected particle size, minimum track length, and blur setting, a camera level of 10 and detection threshold of 5.

Proteome analysis of exosomal preparations

Serum exosome fractions 8, 9 and 10 were prepared for mass spectrometry MS-based proteomic analysis. Proteomes were concentrated using chloroform-methanol precipitation, dissolved in 90% formic acid, their concentrations estimated at 280 nm using a Nanodrop (ND-1000, Thermo Scientific, USA) and aliquots dried using vacuum centrifugation. Proteomes were then processed and quantified as before.[321] Peptides from each fraction were desalted using C18 ZipTips™, concentrations estimated by Qubit quantitation (Invitrogen), dried by vacuum centrifugation and re-suspended in 3% acetonitrile (ACN; v/v)/0.1% formic acid (v/v). Samples (0.5 μg) from exosome elution fractions 8-10 were separated by nanoLC using an Ultimate nanoRSLC UPLC and autosampler system (Dionex) before analysed on a QExactive Plus mass spectrometer (Thermo Electron, Bremen, Germany) as previously described.[321] MS/MS data were analysed using Mascot (Matrix Science, London, UK; v2.4.0) with a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 4.0 PPM. Peak lists were searched against a SwissProt database (2017_11),

selected for *Homo sapiens*, trypsin digestion, maximum two missed cleavages, and variable modifications methionine oxidation and cysteine carbamidomethylation. Exosome proteins were annotated using Vesiclepedia (http://microvesicles.org)[322] and Functional Enrichment Analysis Tool (FunRich; v2.1.2; http://funrich.org)[323].

RNA extraction and small RNA sequencing

Serum exosomes were processed for RNA extraction using the Plasma/Serum Circulating & Exosomal RNA Purification Mini Kit (Norgen Biotek, Cat. 51000) according to the manufacturer's protocol. Extracted total RNA samples were analysed with a Eukaryote Total RNA chip on an Agilent 2100 Bioanalyser (Agilent Technologies, United States) to confirm sufficient yield, quality and size of RNA. Exosome RNA sequencing libraries were then constructed using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (BioLabs, New England) according to the manufacturer's instructions. Yield and size distribution of resultant libraries were validated using Agilent 2100 Bioanalyser on a high-sensitivity DNA assay (Agilent Technologies, United States). Libraries were then pooled with an equal proportion for multiplexed sequencing on Illumina HiSeq. 2000 System at the Ramaciotti Centre for Genomics.

Data pre-processing, differential expression analysis and pathway analysis

Data pre-processing was performed using a pipeline comprising of adapter trimming (cutadapt), followed by genome alignment to human genome hg 19 using Bowtie (18bp seed, 1 error in seed, quality score sum of mismatches <70). Where multiple best strata alignments existed, tags were randomly assigned to one of those coordinates. Tags were annotated against mirBase 20 and filtered for at most one base error within the tag. Counts for each miRNA were tabulated and adjusted to counts per million miRNAs passing the mismatch filter. All samples achieved miRNA read counts >45,000 read counts and miRNAs with low abundance (<50 read counts across more than 20% of samples) were removed. Differential expression analysis was performed using three different statistical hypothesis tests including a non-parametric two-sample Wilcoxon test and two parametric tests: Student's t-test,

and an exact test (implemented in Bioconductor EdgeR), which tests for differences between the means of two groups of negative-binomially distributed counts. Benjamini & Hochberg adjusted *p*-values were also calculated. Data pre-processing and differential expression analysis were performed using Bioconductor and R statistical packages. Pathway analysis was performed using Ingenuity® software (Ingenuity Systems, USA; http://analysis.ingenuity.com). MicroRNA target filters were applied to significant, differentially expressed miRNAs (unadjusted *p*-value≤0.05 in all three statistical methods) and mRNA target lists were generated based on highly predicted or experimentally observed confidence levels. Core expression analyses were performed with default criteria to determine the most significant functional associations (biological and canonical pathways) of mRNAs targeted by dysregulated miRNAs.

Univariate analysis

We performed logistic regression (LR) and receiver operator characteristic (ROC) analysis to assess the predictive power of individual miRNAs between the two groups of interest. LR was used to identify linear predictive models with each miRNA as the univariate predictor. The quality of each model was depicted by the corresponding ROC curve, which plots the true positive rate (i.e., sensitivity) against the false-positive rate (i.e., 1-specificity). The area under the ROC curve (AUC) was then computed as a measure of how well each LR model can distinguish between two diagnostic groups. We then used leave-one-out cross validation (LOO-CV) to estimate the prediction errors of the LR models. LOO-CV learns the model on all samples except one and tests the learnt model on the left-out sample. The process is repeated for each sample and the error rate is the proportion of misclassified samples. Overall, cross validation is a powerful model validation technique for assessing how the results of a statistical analysis can be generalized to an independent dataset.[229] These analyses were performed using R stats (glm) and boot (cv.glm) packages.

Multivariate analysis

To assess the predictive power of multiple miRNAs as disease signatures, samples were first randomly partitioned into two disjoint sets of **discovery** (70% of samples) and **validation** (30% of samples). miRNAs differentially expressed in the discovery

set (i.e., changes increased or decreased by fold change≥2 and unadjusted *p*-value≤0.05 in all three statistical hypothesis tests) were then selected as features/predictors of RF multivariate predictive model. RF is a multivariate nonlinear classifier that operates by constructing a multitude of decision trees at training time in order to correct for the overfitting problem.[324] RF was trained on the discovery set and the resultant predictive model was then used to predict GBM or GII-III patients versus HCs based on the read count values of identified miRNAs in validation samples. For statistical rigour, to account for random partitioning of the samples into discovery and validation sets, the whole process was repeated 100 times. We then chose stable miRNAs—i.e., those identified to be differentially expressed in more than 75% of iterations—as predictors of an RF model using all samples and the out-of-bag (OOB) error was reported as an unbiased estimate of the model predictive power. The 'importance' or relative contribution of each feature (differentially expressed miRNAs) in the RF performance was then estimated based on the 'mean decrease accuracy' measure as detailed by Breiman in 2001.[280] All the analyses were performed using R 'caret' and 'RandomForest' packages.

Data availability

Exosomal miRNA raw data will be accessible at NCBI Gene Expression Omnibus (GEO; **accession number to be provided**). In the interim, the miRNA sequencing data is available at: https://github.com/VafaeeLab/glioblastoma_exosomal_miR_markers.

Results

Characterisation of serum exosomes isolated prior to miRNA sequencing

Serum exosomes were isolated by size exclusion chromatography. The combined elution fractions 8-10 showed particle sizes with a mean diameter 89.1 ± 2.5 nm and modal diameter of 81.7±5.5 nm *(Figure 4-1A)*. TEM confirmed the presence of similarly sized particles with vesicular morphologies, characteristic of exosomes *(Figure 4-1B)*. MS analysis confidently identified 1167, 861 and 636 proteins in qEV elution fractions 8, 9 and 10 from healthy serum, respectively. Overall, 87 of the top

100 proteins commonly identified in exosomes were confidently sequenced across the three fractions, including all top 10 exosomal proteins *(Figure 4-1C-1)*. Primary sub-cellular localisations included significant enrichments of 'exosome' and 'blood microparticle' related proteins across all fractions, with minimal contamination from other compartments, including the nucleolus *(Figure 4-1C-2)* where certain miRNAs show specific nuclear enrichment.[325] Prior to RNA extraction, serums were treated with RNaseA to remove circulating RNAs that may confound measurements of exosomal RNAs.[270] RNA extracted from each sample yielded profiles typical for exosomes, showing an absence of ribosomal RNA and enrichment of small (<200 nt) RNA species *(Figure 4-1D)*.

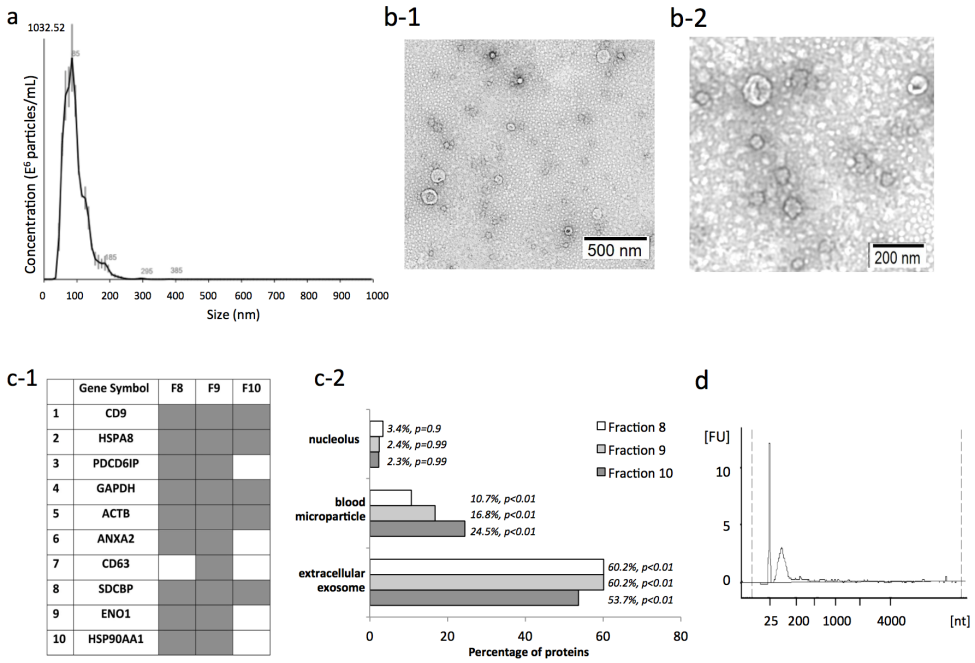


Figure 4-1 Characterisation of serum exosomes isolated in fractions 8-10 by size exclusion chromatography prior to miRNA sequencing

A) Size distribution of particles as analysed by NTA. B) Transmission electron microscopy allowed visualization of vesicles with sizes ranging from 60-110 nm in diameter, scale bars = 500 nm (B-1, *wide field*) and 200 nm (B-2, *close-up*). C-1) Mass spectrometry-based proteome analysis of size chromatographic elution fractions 8-10 identified all top 10 exosome marker proteins and (C-2) showed significant enrichment of proteins characteristic of exosomes and blood microparticles. Proteins identified in fractions 8-10 showed limited, non-significant associations to compartments like the nucleolus, where certain miRNA species are concentrated. D) Bioanalyser trace of RNA extracted from serum exosomes shows the main population of small RNA and no ribosomal RNA.

Differentially expressed exosomal miRNAs in GBM patient sera

Circulating exosomal miRNA profiles from patients with histopathologically confirmed IDH^{WT} GBM (n=12) were compared to age- and gender-matched HCs (n=12; see *Table 4-1* for discovery cohorts; *Table 4-2* for validation cases). We employed three statistical approaches (Student's t-test, Fisher's exact, Wilcoxon rank sum) to identify a discovery set of differentially expressed miRNA biomarkers. miRNA biomarkers were identified if their differential expression met a fold change≥2 in either direction and unadjusted p-values≤0.05 in all statistical tests applied. Using this approach, we identified 26 miRNAs significantly dysregulated between HCs and GBM patients (*Table 4-3*; *Figure 4-3A*).

Table 4-1 Overview of cohorts used for discovery miRNA analyses

	GBM, IDH^{WT}	**GBM-matched HC**	**GII-III, IDH^{MUT}**	**GII-III-matched HC**
Sample *n*	12	12	10	10
Age (mean ±SD)	63.3 ± 11.5	56.2 ± 12.4	42.9 ± 12.7	42.7 ± 10.2
Gender	7M, 5F	7M, 5F	6M, 4F	6M, 4F

Table 4-2 Additional patients and cohorts used for validation

Patient/cohort	Age	Gender	Diagnosis	Notes
GBM1_relapse	46	M	GBM IV	Pre-operative blood taken after recurrence of GBM1 (8-month relapse)
GBM12_prior	45	F	GBM IV	Pre-operative blood taken before removal of earlier GBM lesion (GBM12; 4.6 months prior)
GBM13	33	M	GBM IV	Glioblastoma, IDH^{MUT}, WHO (2016) grade IV
GBM14	56	M	High-grade glioma	No surgery/tissue pathology performed, diagnosis based on repeat MRIs. Overall survival of 8.1 months.
GI_C	24	F	Ganglioglioma grade I	$GFAP^{+}$ in glial component/ $NeuN^{+}$ in neuronal component, $IDH1^{WT}$, $ATRX^{+}$, $BRAF(V600E)^{+++}$
HC (*n*=9)	36.2± 10.3	5F, 4M	Healthy controls	–
MS_C (*n*=9)	35.3±10 .4	5M, 4F	Relapse-remitting Multiple Sclerosis	All patients had active lesions; were untreated (*n*=5) or receiving different immunomodulatory therapies (*n*=4)

Abbreviations: F, female; GBM, glioblastoma; GII-III, glioma grade II-III; GI_C, ganglioglioma grade I control case; HC, healthy controls; M, male; MS_C, multiple sclerosis control cohort. (Mean age with standard deviation is provided for each cohort.)

Table 4-3 Significant dysregulated miRNAs in serum exosomes from glioblastoma (GBM) patients (n=12) relative to healthy controls (HC; n=12)

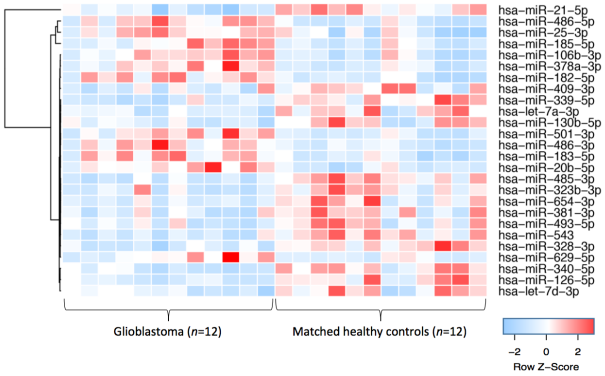
miR-	CPM (GBM)	CPM (HC)	FC	Exact test	t-test	Wilcoxon	Error rate	AUC
486-5p	25291.6	8522.6	3.0	1.6E-07*	4.0E-04*	1.0E-04*	0.149	0.924
182-5p	2090.5	850.6	2.5	5.7E-07*	3.0E-04*	2.0E-04*	0.151	0.917
486-3p	277.4	114	2.4	5.0E-06*	0.002*	3.0E-04*	0.149	0.910
378a-3p	2083.2	875.2	2.4	1.4E-06*	0.003*	4.0E-04*	0.158	0.903
183-5p	645.8	267.9	2.4	2.0E-05*	0.001*	0.001*	0.176	0.882
501-3p	359.6	157.3	2.3	1.1E-05*	0.002*	0.001*	0.161	0.875
20b-5p	594.6	266.3	2.2	2.9E-06*	0.002*	1.0E-04*	0.133	0.938
106b-3p	2703.2	1215	2.2	3.9E-06*	0.001*	0.001*	0.160	0.889
629-5p	896.8	415	2.2	0.001*	0.047	0.04	0.235	0.743
185-5p	23250.5	11424.1	2.0	4.3E-05*	0.007*	0.005*	0.207	0.833
25-3p	21838.8	10949.9	2.0	0.001*	0.002*	0.006*	0.199	0.826
21-5p	73535.3	142796.9	-2.0	2.7E-04*	4.2E-05*	5.0E-05*	0.133	0.944
7a-3p	82.1	176.3	-2.0	0.003*	0.005*	0.010*	0.187	0.806
381-3p	190.5	397.9	-2.0	0.009*	0.012	0.012	0.220	0.799
409-3p	1146.9	2242.5	-2.0	0.019	0.029	0.024	0.233	0.771
7d-3p	1050.5	1912.9	-2.0	0.005*	0.013	0.017	0.209	0.785
323b-3p	117.3	288.3	-2.4	0.004*	0.010*	0.004*	0.199	0.840
328-3p	382.5	922.5	-2.5	4.6E-06*	2.0E-04*	2.2E-05*	0.117	0.958
339-5p	90.1	234.8	-2.5	1.2E-06*	2.0E-04*	3.3E-05*	0.109	0.951
340-5p	1536	3848.1	-2.5	4.8E-06*	1.0E-04*	5.0E-05*	0.134	0.944
126-5p	1222.3	2947	-2.5	5.6E-06*	0.002*	0.001*	0.150	0.896
130b-5p	111.9	248.9	-2.5	0.007*	0.009*	0.024	0.203	0.771
493-5p	210	514.4	-2.5	0.010*	0.015	0.028	0.221	0.764
543	223.1	753.2	-3.3	2.5E-06*	3.0E-04*	2.0E-04*	0.143	0.917
654-3p	110.2	342.5	-3.3	2.2E-04*	0.009*	0.006*	0.193	0.826
485-3p	93.2	352.3	-3.3	5.8E-07*	1.0E-04*	3.3E-05*	0.123	0.951

Abbreviations: CPM, miRNA counts per million; FC, fold change; error rates estimated by leave-one-out cross validation; AUC, area under the receiver operating characteristic; Significant Benjamini & Hochberg adjusted *p*-values are indicated by asterisks.

Functional analysis of dysregulated miRNAs in GBM

We explored biological and canonical pathways associated with exosomal miRNAs changing in GBM patient sera relative to HCs. The identities of 44 miRNAs (p-value≤0.05 in all three tests; no fold change restriction) were uploaded into the Ingenuity Pathway Analysis environment to analyse molecular pathways overrepresented in their targets. The dysregulated miRNAs target mRNAs that are significantly associated with 'cancer' ($1.96E^{-06}$<p-value<$1.52E^{-16}$) and 'neurological disease' ($1.72E^{-06}$<p-value<$8.76E^{-13}$) with around half of targeted mRNAs implicated in GBM (p-value=$3.36E^{-12}$) and glioma signaling pathways (p-value=$1.25E^{-09}$; *Figure 4-2B, C*).

a.



b.

Top canonical pathways	p-value	Overlap
Molecular mechanisms of cancer	$2.16E^{-12}$	39.2% (152/388)
Glioblastoma multiforme signaling	$3.36E^{-12}$	48.4% 78/161)
Pancreatic adenocarcinoma signaling	$6.07E^{-11}$	50.8% (61/120)
Role of macrophages, fibroblasts & endothelial cells in Rheumatoid arthritis	$4.37E^{-10}$	39.3% (119/303)
Glioma signaling	$1.25E^{-09}$	49.6% (56/113)

Diseases and disorders	p-value	#Molecules
Cancer	$1.96E^{-06}$ – $1.52E^{-16}$	4322
Organismal injury and abnormalities	$1.97E^{-06}$ – $2.97E^{-13}$	4371
Neurological disease	$1.72E^{-06}$ – $8.76E^{-13}$	785
Tumor morphology	$1.96E^{-06}$ – $2.81E^{-12}$	366
Developmental disorder	$1.39E^{-06}$ – $3.49E^{-12}$	601

Molecular and cellular functions	p-value	#Molecules
Cell death and survival	$2.13E^{-06}$ – $8.28E^{-17}$	1469
Gene expression	$8.34E^{-07}$ – $1.67E^{-15}$	1010
Cellular growth and proliferation	$1.92E^{-06}$ – $5.23E^{-15}$	1466
Cell cycle	$2.01E^{-06}$ – $6.25E^{-15}$	671
Cellular development	$1.92E^{-06}$ – $1.51E^{-14}$	1579

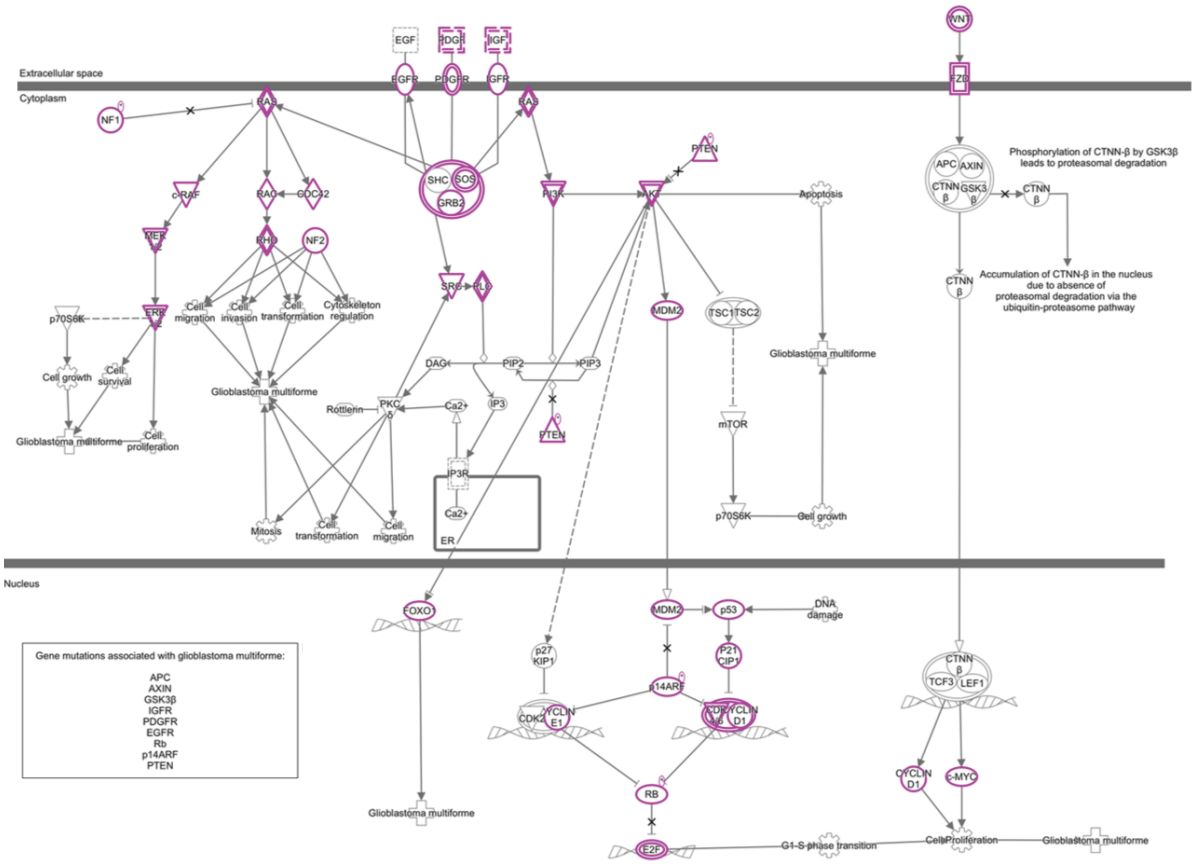


Figure 4-2 Differentially expressed miRNAs and their pathway analyses

A) Hierarchical clustering of 26 differentially expressed miRNAs shows clear separation of GBM patients and HC exosomal profiles (fold change≥2 or ≤0.5; unadjusted *p*-values≤0.05 in all three statistical tests). B) Functional pathway analysis of mRNAs targeted by 44 significantly changing miRNA (unadjusted *p*-values≤0.05 in all three statistical tests) in GBM circulating exosomes. Top canonical pathways, diseases and disorders and molecular and cellular functions are listed with the numbers of overlapping molecules and significance of associations (right-tailed Fisher exact test, *p*-value). C) Ingenuity Pathway Analysis revealed significant overlaps with Glioblastoma signaling pathway (*p*-value=3.36E-12). Glioblastoma signaling pathway annotated with molecules targeted by significant, differentially expressed exosomal miRNAs (in magenta).

Selection of signature miRNA classifiers for preoperative GBM diagnosis

The predictive power of each miRNA was estimated using LR models, in which individual miRNA expression profiles were used as predictors. ROC curves were determined and AUC measures were ≥0.74 across the 26-dysregulated miRNAs (*Table 4-3; Figure 4-3*).

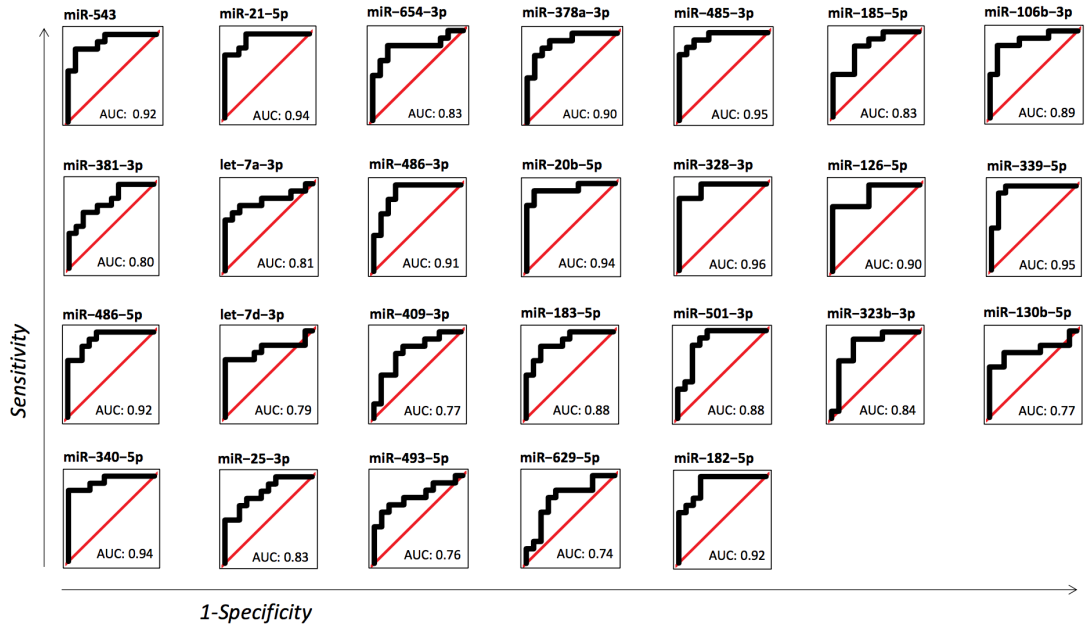
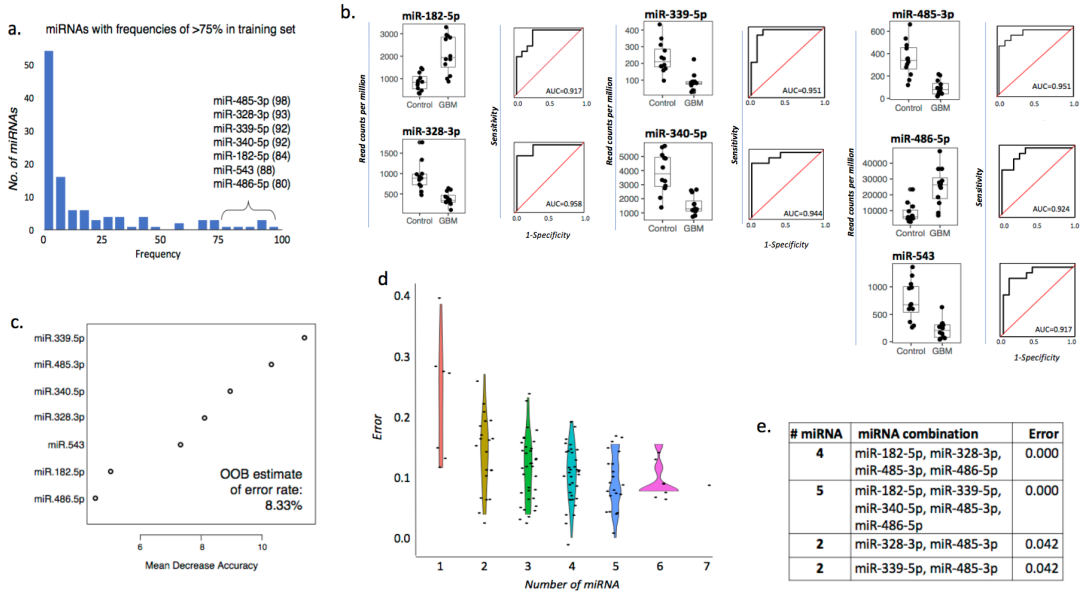


Figure 4-3 ROC curves of differentially expressed miRNAs in GBM versus HCs

In silico validation by LOO-CV correctly identified the test sample on average 83% of the time (range 77–89%). We then used partitioning (70% training and 30% test) and RF multivariate modeling to determine whether expression patterns of a subset of differentially expressed miRNAs could improve the predictive power. Using these methods, seven miRNAs (miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p and miR-543) distinguished GBM patients from healthy subjects in more than 75% of the random data partitions and were selected as the most 'stable' miRNA classifiers *(Figure 4-4A and B)*. The RF model was repeated using all iterations of the seven most stable miRNAs and achieved an overall predictive power of 91.7% for classifying GBM patients from HCs *(Figure 4-4-C and D)*. Strikingly, within this model, several miRNA combinations were able to distinguish GBM patients from HCs with perfect accuracy, including a panel of four

miRNAs (miR-182-5p, miR-328-3p, miR-485-3p, miR-486-5p) and five miRNAs (miR-182-5p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p; *Figure 4-4E*).



# miRNA	miRNA combination	Error
4	miR-182-5p, miR-328-3p, miR-485-3p, miR-486-5p	0.000
5	miR-182-5p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p	0.000
2	miR-328-3p, miR-485-3p	0.042
2	miR-339-5p, miR-485-3p	0.042

Figure 4-4 Selection of signature miRNAs

A) miRNAs appearing in >75 of 100 partitions (70% training set, 30% test set) were selected as the most stable miRNA classifiers by Random Forest modelling (frequencies are specified in brackets). B) Box-and-whisker plots and ROC curves with AUC calculations demonstrate the individual discriminatory power of the seven most stable miRNA classifiers. C) miRNAs were ordered by the importance of their contribution to discriminating GBM from HCs; overall OOB error rate of the seven features was 8.33%. D) RF model was performed again using all possible combinations of seven most stable miRNAs to find combinations (i.e., signatures) with the highest multivariate predictive power. Error rates of different combinations were stratified by the number of miRNAs (signature size) and their distributions were displayed as violin plots. E) miRNA combinations that discriminate between GBM and HCs with the highest accuracy.

To assess the temporal stability of the GBM miRNA signature in the same patients, we tested preoperative sera collected at a GBM recurrence (GBM1 patient relapsed and required additional surgery after eight months) and from an earlier GBM lesion (excised 4.6 months before GBM12; *Table 4-2*). Using the panel of seven exosomal miRNAs, both GBM1-*relapse* and GBM12-*prior* were classified as GBM, in line with diagnostic histopathology. We also tested two independent samples, including a patient diagnosed with IDH^{MUT} GBM (GBM13) and a patient diagnosed with 'high-grade glioma' based on repeat MRIs and overall survival of 8.1 months (GBM14; see

Table 4-2). Both GBM13 and GBM14 were classified as GBM using the miRNA panel.

To further test the specificity of the GBM miRNA signature, we assessed its ability to distinguish GBM patients from additional healthy subjects and non-glioma disease controls. The panel accurately classified all additional healthy subjects (*n*=9; *Table 4-2*) as well as a patient with ganglioglioma WHO (2016) grade I, a slow-growing, benign brain tumour with glioneuronal components (GIC-1). Next, we assessed the impact of neuroinflammatory disease processes on the specificity of our exosomal miRNA panel ability. The bioinformatics analysis above showed that dysregulated miRNAs also target mRNAs significantly associated with autoimmune rheumatoid arthritis and broadly to 'neurological disease' *(see Figure 4-2B)*. Our GBM miRNA panel was used to discriminate patients with the inflammatory autoimmune disease, MS. Sera were sampled from MS patients with active gadolinium enhancing demyelinating lesions, either untreated or receiving immunomodulatory therapies (*n*=9; *Table 4-2*). All MS patients were classified as controls, indicating the robustness of our exosomal miRNA signature for GBM identification.

miRNAs dysregulated in IDH-mutant II-III gliomas provide additional markers for glioma severity and IDH mutational status

We then compared serum exosome miRNA profiles between IDH^{MUT} grade II-III glioma patients (*n*=10; mean age=42.7) and matched HCs (n=10; mean age=42.9; see *Table 4-1*) and identified 23 differentially expressed miRNAs (fold change≥2; unadjusted $p<0.05$ in all three tests. Of these, 12 miRNAs were shared with the GBM analysis and showed the same direction of change (*Figure 4-5A*).

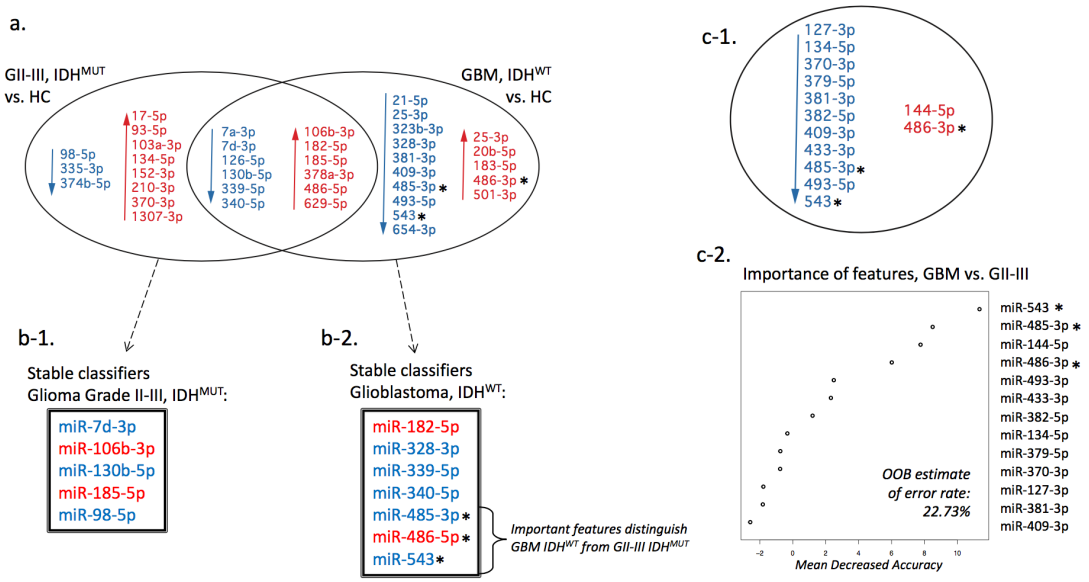


Figure 4- 5 Representing stable classifiers for glioma tumour grades II-III (IDH^{MUT}) and glioblastoma (IDH^{WT})

a) A Venn diagram summarises the differentially expressed miRNAs between IDH^{MUT} glioma tumour grades II-III (GII-III; *n*=10), IDH^{WT} glioblastoma (GBM; *n*=12) and corresponding age- and gender-matched healthy controls (HC; fold change≥2 or ≤0.5; unadjusted *p*-values≤0.05 in all three statistics tests, i.e., exact, t-test and Wilcoxon), with 12 overlapping differentially expressed miRNAs. Decreased expression is indicated in blue and increased expression in red. The most stable miRNAs for classifying (b-1) GII-III IDH^{MUT} and (b-2) GBM IDH^{WT} from HCs are listed and show distinct features. (c-1) Summary of differentially expressed miRNAs between the GBM IDH^{WT} and GII-III IDH^{MUT} cohorts and (c-2) plot of 'importance' of each individual miRNA for discriminating GBM from GII-III; OOB error rate is 22.73%. Three of the top four features that distinguish GBM IDH^{WT} from GII-III IDH^{MUT} were only identified in the GBM vs. HC comparative analysis, are members of the GBM miRNA signature that together accurately classify GBMs from HCs and may be specific markers for GBM (indicated by asterisks in a, b-1, c-1, and c-2).

AUC curve measures were ≥0.78 (average 0.88) across the 23 dysregulated miRNAs, and LOO-CV correctly identified the test sample on average 83% of the time (range 77–88%; *Figure 4-6*).

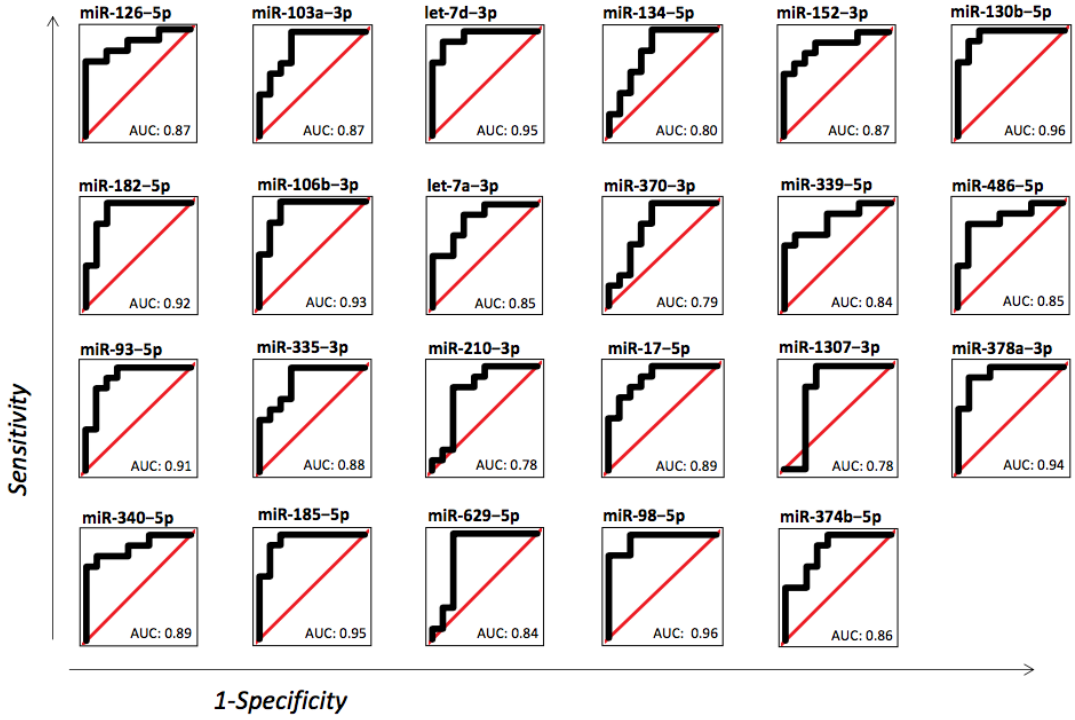


Figure 4-6 ROC curve of differentially expressed miRNAs between grades II-III glioma versus controls

RF modeling performed on partitioned data selected miR-7d-3p, miR-98-5p, miR-106b-3p, 130b-5p and 185-5p as the most stable features for classifying grade II-III glioma patients from healthy participants, with a predictive power of 75.0% (*Figure 4-5C and Figure 4-7*). The most stable miRNAs for classifying GII-III IDH^{MUT} from HCs were distinct from GBM IDH^{WT} signature miRNAs (*Figure 5-5 –b1, b2*).

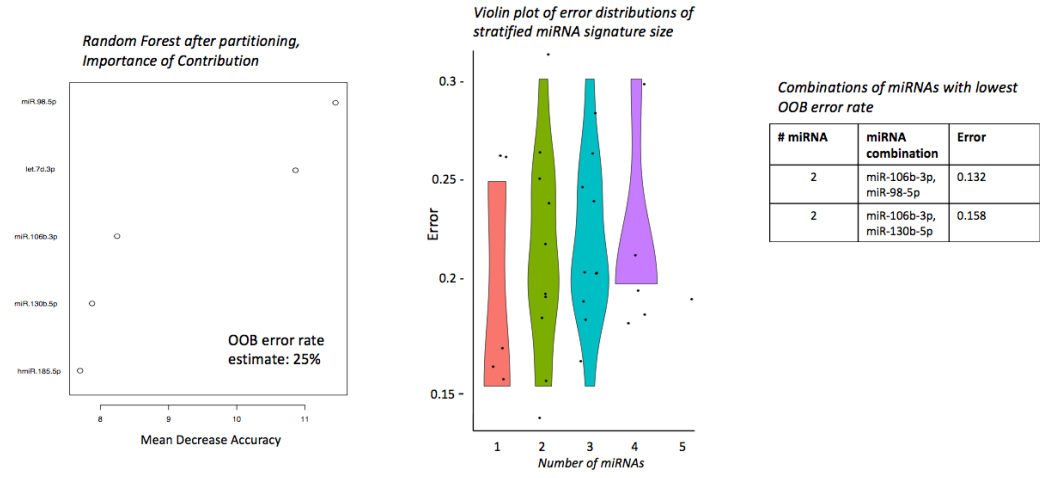


Figure 4-7 Partitioning and RF modelling to select stable miRNAs for grade II-III glioma versus matched HCs

The sncRNA data was further interrogated to ascertain whether a subset of miRNAs showed potential for distinguishing glioma disease severity or IDH mutational status. Direct comparisons between GBM IDH^{WT} and GII-III IDH^{MUT} patients revealed 13 differentially expressed miRNAs (fold change≥2; unadjusted $p<0.05$ in all three tests; (*Figure 4-5C-1*). AUC curve measurements were ≥0.78 (average 0.84) across the 13 dysregulated miRNAs and LOO-CV correctly identified the test sample on average 80% of the time (range 76–86%). Numbers of significant miRNA were too few to perform partitioning, so a single RF model was constructed from all 13 dysregulated miRNAs that showed an estimated predictive power of 77.4% (*Figure 4-5C-2*) Interestingly, three of the top four features that discriminate GBM IDH^{WT} from GII-III IDH^{MUT} are members of the GBM miRNA signature (i.e., miR-543, miR-485-3p and miR-486-3p), changing only in GBM patient sera relative to healthy participants (indicated by asterisks in *Figure 4-5*).

Discussion

Using unbiased high-throughput next generation sequencing and an integrative bioinformatics pipeline;[270] we have identified differentially expressed serum exosomal miRNAs that discriminate GBM patients from HCs. Machine-learning approaches on miRNAs were used to examine their individual and shared predictive abilities for a pre-operative GBM diagnosis via a blood test. Of the 26 differentially expressed miRNAs in GBM patients' relative to HCs, we selected a stable signature panel of seven miRNAs. Together, expression levels of miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p and miR-543 predicted a preoperative GBM diagnosis with 91.7% accuracy. Within this multivariate model a combination of just four miRNAs (miR-182-5p, miR-328-3p miR-485-3p miR-486-5p) distinguished GBM patients from HCs with perfect accuracy (100.0%).

There have been multiple studies examining 'free-circulating' miRNAs in glioma patients with varying success. A recent meta-analysis of these studies found the specificity and sensitivity of circulating miRNAs was 0.87 and 0.86, respectively, while noting the large heterogeneity of circulating miRNAs within the included studies.[326] The heterogeneity is likely due to differences in data normalisation used in

quantitative reverse transcriptase PCR studies, with no universally accepted endogenous housekeeping control.[326] Interestingly, the majority of miRNAs identified in our exosomal signature have not been previously identified in 'free-circulating' studies. This is consistent with the notion that exosomes represent a distinct pathway of nucleic acid release from cells, and contain selectively packaged miRNA species.[255] We have previously shown the effects of RNAse pre-treatment of serum prior to exosome isolation, as performed in this study, drastically alters the miRNA profiles identified, presumably due to eradication of co-precipitated 'free-circulating' miRNAs.[270] Moreover, normalisation of deep sequencing data is not dependant on comparison to a reference signal or housekeeping gene, potentially reducing variability in data analysis.

Functional pathway analysis of mRNA species targeted by exosomal miRNAs dysregulated in GBM patient sera showed highly significant associations to specific GBM molecular pathways. This provides confidence that the miRNA biomarkers resolved by our methods are relevant to this particular disease setting. Previous studies have identified roles for all seven GBM miRNA classifiers in various aspects of glioma and GBM biology. miR-182, detected here in significantly higher levels in GBM sera, was proposed as a marker of glioma progression, critical for glioma tumourigenesis, tumour growth and survival *in vitro*,[327,328] with high miR-182 tissue expression observed in GBM[329] and associated with poor overall survival[330]. Also in line with observations here, the up-regulation of miR-486 was shown to promote glioma aggressiveness both *in vitro* and *in vivo*.[331] Exosomal miRNAs identified with lower expression levels in GBM patient sera are also substantiated by the literature. Functional assays indicate tumour suppressive roles of miR-328[332], miR-340,[333,334] miRNA-485-5p[335] and miR-543[336] with low levels observed in tumour tissues relative to normal brain[332,334-336] and low tissue expression levels significantly associated to poor patient outcomes [332,334]. While miR-339 (decreased levels in GBM patients here) was shown to contribute to immune evasion of GBM cells by modulating T-cell responses,[337] inhibitory roles for miR-339 were reported in acute myeloid leukemia,[338] hepatocellular carcinoma,[339] gastric,[340] colorectal,[341] breast[342] and ovarian cancers[343].

The GBM miRNA signature was able to accurately classify all additional specimens in the validation sets (healthy, *n*=9; non-glioma, *n*=10), including patients with gadolinium enhancing active demyelinating lesions. Tumefactive demyelination is a well-recognised mimic of GBM.[344] The GBM signature also correctly classified four additional GBM specimens, including two serial collections from patients within the discovery cohort as well as two independent patients. Further testing is needed to determine whether the miRNA panel can reliably diagnose GBM in large, independent patient cohorts. Moreover, the correlation between a positive GBM classification and tumour burden needs to be addressed. To this end, longitudinal studies should be pursued to assess whether the GBM miRNA panel can detect time critical GBM tumour recurrences.

There is more than one pathological route to a GBM; primary and secondary GBMs are distinct entities with IDH mutations considered a genetic signpost.[345] The only patients where early detection of a GBM tumour is likely are arguably those with diffuse and anaplastic (grade II-III) gliomas who progress with a secondary GBM recurrence (IDH^{MUT}). Accordingly, the identification of reliable and readily accessible circulating progression markers is an important step towards precision medicine for patients diagnosed with low-grade gliomas. While the GBM miRNA signature was described in serum exosomes from IDH^{WT} GBM patients, it was also able to categorise a patient with IDH^{MUT} GBM (GBM13) from healthy participants. It is worth noting that miRNA members of the GBM signature panel (specifically, increased miR-182-5p, decreased miR339-5p and miR-340-5p) were also identified in the IDH^{MUT} GII-III comparative analysis. Whether these miRNA changes are related to IDH mutational status, glioma grade, or a combination of the two, cannot be delineated here. However, our multivariate modeling did identify distinct panels of miRNAs for classifying GBM and glioma patients from their corresponding matched healthy control cohorts. Moreover, three GBM signature panel miRNAs that were unique to the GBM vs control comparative analysis (increased miR-486-5p and decreased miR-485-3p and miR-543) were among the top four features that distinguish GBM IDH^{WT} from GII-III IDH^{MUT} and therefore, might be specific for GBM IDH^{WT} (indicated by asterisks in *Figure 4-4*). These encouraging results demonstrate the potential for exosomal miRNA profiles to be used for glioma

subtyping and grading, including the determination of mutational states. Expansion of these discovery analyses to include well defined cohorts of glioma subtypes with sufficient *n,* will likely resolve biomarkers of more nuanced specificity.

Chapter 5:
Concluding remarks and future directions

Conclusions

Circulating exosomes in body fluids display a variety of protein and RNA contents in healthy individuals and patients with a different disease, and these can be measured to assess their potential as biomarkers.[346] It has been reported that patients with glioblastoma can be distinguished from healthy individuals based on the increased level of EGFRvIII mRNA in tumour-derived exosomes.[347] Yet another study reported that the presence of EGFR localized to exosome membranes could be useful as a biomarker for lung cancer diagnosis.[348] In pancreatic cancer patients, the presence of proteoglycan glypican-1 positive exosomes not only distinguishes them from healthy individuals and patients in the benign stage of cancer[349] but also the level of these exosomes correlated with tumour burden and survival of pre- and post-surgical patients. It has been reported that plasma microvesicles released by prostate acinar cells represents a novel markers for prostate cancer and proteomics profiling of EVs identified candidate markers of the disease.[350]

Exosomes have also been evaluated as a potential biomarker for other non-cancer diseases of multiple organs including lung,[351] arteries,[352] kidney,[353] liver,[354] and the central nervous system (CNS). The serum exosomal miRNA profiles from broncho-alveolar lavage fluids of asthmatic patients differed them from healthy individuals.[351] In cardiovascular disease, significantly differentially expressed miR-192 in serum exosomes predicted the subsequent development of heart failure in patients after acute myocardial infarction.[355] Also in patients with hepatocellular carcinoma, recurrence of tumour after liver transplantation correlated to increased levels of miRNAs in serum exosomes.[356] In the CNS, extracellular accumulation of

abnormally processed tau protein results in tau-induced neurodegeneration. It has been reported that in the M1C neuroblastoma tauopathy model, exosomes are enriched in AT270 phospho-tau, can be used as a valuable biomarker for early Alzheimer disease.[357] Profiling proteomics of serum circulating exosomes identified a variety of differentially abundant proteins as a biomarker to discriminate patients with Parkinson or Alzheimer diseases from healthy individuals.[358] For instance, the level of autolysosomal proteins in serum exosomes distinguished patients with pre-clinical Alzheimer disease from healthy individuals and patients with frontotemporal dementia.[359] Furthermore, Bellingham and his colleagues reported that a small RNA deep sequencing of neuronal exosomes contained a diverse range of RNA species, in particular, a distinct miRNA signature that could be utilised for diagnosing neurodegenerative disorders.[360]

In this thesis, I tested the hypothesis that circulating exosomes and their RNA cargo can be exploited as accessible and informative biomarkers for two disparate CNS pathologies: multiple sclerosis (MS) and glioblastoma. The presence of the blood-brain barrier (BBB) that protects the CNS makes brain chemistry difficult to monitor, and currently available techniques are invasive and costly. A blood-based biomarker test based on exosomes that can cross the BBB will facilitate monitoring CNS diseases longitudinally in a cost-effective and minimally invasive manner.[142,224] Exosomes are enriched with selectively packaged RNA from their producing cell, and thus by interrogating the exosomal RNA from the serum of patients and control subjects, I was able to determine that serum exosome miRNA signatures not only reflect the presence of a pathological CNS condition, but can also indicate the subtype of CNS pathology, and in some cases predict treatment response.

In chapter 2, I examined a clinical cohort to ask whether serum exosomal miRNA profiles might be useful as disease biomarkers. By comparing serum exosomal miRNA profiles among healthy control and MS patients with varying disease activity I found a core signature of four miRNAs that was able to discriminate healthy individuals from MS patients. I also identified and validated nine miRNAs within MS patients that were capable of differentiating MS patients with different states of disease activity: relapsing-remitting MS (RRMS) or secondary progressive

MS/primary progressive MS (SPMS/PPMS). This is important because it implies that, in addition to disease diagnosis, serum exosomal miRNAs may act as tools for monitoring disease progression and allow for the early detection of disease resulting in better control of disease symptoms and ultimately improving quality of life.

Interestingly, the majority of serum exosomal miRNA biomarkers were novel in that they have not been previously reported to be associated with MS. These findings suggest that serum exosomes are a unique source of biomarkers that have the potential to reveal signatures of disease that may not be visible by examination, for example, of total free circulating miRNA. Taken together, the results of this chapter revealed that serum exosomal miRNA can be employed to diagnose MS, and furthermore is able to classify MS patient disease activity.

In chapter 3 I focused on determining whether serum exosome miRNA signatures might reveal something about the efficacy of Fingolimod, a disease modifying treatment (DMT). Although Fingolimod is an oral immunomodulator for MS therapy[263] with an advantage over other injectable DMTs with no side-effect due to injection, its efficacy must be monitored on a case–by–case basis to manage an individual treatment strategy. My findings in chapter 2 suggest that serum exosomal miRNA signatures can reflect MS disease status, and differentiate between patients with RRMS and SPMS/PPMS. Therefore, I first sought to identify molecular biomarkers that indicate the active phase of RRMS and the efficacy of Fingolimod treatment in RRMS patients. I identified 15 dysregulated miRNAs that individually could discriminate between patients in relapse and those in the remitting phase six months after treatment. Current monitoring of RRMS patients requires regular MRI scanning (6 -12 monthly) which is costly and difficult to access for patients living in rural or remote communities. Thus having blood-based biomarkers will assist in confirming the diagnosis and allow for closer monitoring of disease activity. Using machine-learning approaches, the 15 miRNAs were refined to a set of 11 with a 92% accuracy rate of predicting active from quiescent RRMS phase. The predicted gene targets of these dysregulated miRNAs suggest that they target genes and biological processes previously associated with MS activity, and this, together with the correlation between presence of the markers and disease progression as determined by gadolinium-based contrast agents for MRI (Gd-MRI) scans, supports the validity

of these unique biomarkers. My findings suggest that circulating exosomes and their RNA cargo may serve as signatures of disease activity/progression that can be used to complement or even potentially replace Gd-MRI scans.

Next, I investigated the pharmacogenomic roles of miRNAs in response to Fingolimod therapy. By comparing the serum miRNA exosome profiles of quiescent *vs* active RRMS patients (as determined by Gd-MRI scan results), I identified 11 miRNAs upregulated in the **stable responders** group (i.e., patients who were quiescent at baseline and six months after treatment) and five miRNAs dysregulated in the **positive responders** (i.e., patients who were active at baseline and become quiescent after six months of treatment). Collectively, these miRNAs were functionally associated with six distinct pathways implicated in MS pathogenesis and relevant to Fingolimod mechanisms: the immune system, the nervous system, signal transduction, lipid metabolism, diseases, and cell cycle regulation. These results suggest that serum exosomal miRNAs are associated with MS immuno-pathogenesis and the molecular mechanism of Fingolimod and thus can be exploited as a biomarker to monitor the activity statuses of RRMS patients and efficacy of therapy.

In chapter 4, I changed disease paradigms and sought evidence to support the idea that investigated exosomal miRNAs can act as biomarkers to diagnose and monitor glioblastomas. This was motivated not only by the urgent need for better monitoring of this devastating illness, but also to further explore the applicability of my serum exosomal miRNA biomarker discovery pipeline, established with MS in chapters 3 and 4. By comparing serum exosomal miRNA profiles between wildtype isocitrate dehydrogenase isoforms (IDH^{WT}) GBM patients and their matched healthy controls, I identified 26 miRNAs differentially expressed between the two. These miRNAs demonstrated a significant association with pathways in cancer, neurological disease and importantly glioma-signalling, indicating their potential to be employed as GBM biomarkers. I used *in silico* validation and partitioning to reduce the number of informative miRNAs to a stable signature set of seven. Testing this panel on another independent sample set of GBM patients with different disease status, MS patients, and healthy controls (HCs), supported the utility of this exosomal miRNA signature for GBM diagnosis.

Chapter 4 also explored whether the serum exosomal miRNA profiles could be used to discriminate low-grade mutant IDH (IDH^{MUT}), grade II-III, glioma patients from matched HCs. Patients with diffuse and anaplastic (grade II-III) gliomas may progress with a secondary GBM recurrence (IDH^{MUT}) as another pathological route to develop GBM. I identified 23 differentially dysregulated miRNAs, which after random forest (RF) modelling and partitioning, was reduced to a stable signature panel of five miRNAs that could distinguish between healthy and low-grade glioma patients. Interestingly these five miRNAs were distinct from the seven GBM IDH^{WT} signature miRNAs, suggesting that miRNAs may be able to act as markers of glioma severity and IDH mutational status.

To confirm this, I directly compared the miRNA profiles of GBM IDH^{WT} and GII-III IDH^{MUT} serum exosomes. While 13 dysregulated miRNAs were identified as potential biomarkers for the differentiation of GBM from glioma, this was not enough for partitioning analysis, and thus a larger independent patient cohort is required for confirmation. The results I achieved however show that serum exosomal miRNA signatures have utility as biomarkers for GBM diagnosis.

Limitations of this work and future directions

Significant effort has been devoted to improving the diagnosis of diseases by discovering miRNA-based biomarkers. However, fundamental challenges arise for the translation of circulating miRNAs in body fluids from bench to patient care.[95] In many studies, a single miRNA has been introduced as a biomarker for the diagnosis of a specific disease. For instance, miR-21 has been reported as a biomarker in many diseases, while further research indicates that miR-21 is a potential biomarker in solid cancers[361] and may even serve as a general disease marker. Hence, signatures that consist of multiple miRNAs seem to be more robust biomarkers to improve differentiation between pathologies, and reflect the complexity of disease phenotypes.[95] Yet, non-concordant results have been obtained for miRNA signatures even within a particular disease. This may be due to variations in sample handling, patient recruitment criteria, profiling techniques, and statistical analysis.[169] Therefore, following the discovery phase, miRNA signatures should be validated in

independent cohorts.

While I developed an efficient protocol to capture the complete profile of serum exosomal miRNAs for use in the diagnosis and monitoring of CNS diseases, due to time and financial limitations my technical choices were limited. There are different biophysical and biochemical properties such as size, density, shape, charge and antigen exposure that can be used to isolate extracellular vesicle (EVs)[362] and each has advantages and disadvantages. Although size-exclusive chromatography (SEC) separates the majority of small EVs and removes almost all of the soluble serum and plasma proteins,[68] it pools EVs originating from a variety of cell types. Perhaps it would be worthwhile to combine some immunoaffinity capture with SEC in order to improve the sensitivity and remove any potentially contaminating exosomes. In general, despite numerous studies on exosomal biomarkers in association with various diseases, due to methodological differences, there is a poor match in the results of individual studies.[363]

The data presented in chapter 2 supported the ability of exosomal RNAs to act as blood-based biomarkers to diagnose MS patients and distinguish RRMS from progressive forms of the disease. Although, the discovery set of biomarkers was validated, this is an initial investigation. In order to assess the clinical utility of predictive biomarkers, more samples and well-controlled prospective clinical trails with appropriate justification should be pursued.

The results of chapter 3 demonstrate that exosomal miRNAs can monitor disease activity and identify patients who respond to Fingolimod therapy. However the high efficacy of Fingolimod therapy and the limited sample size resulted in too few non-responder patients to usefully quantify the predictive power. Longitudinal studies on a larger cohort of RRMS with longer follow up and more time points are needed to define standardised relationships between therapeutic intervention and response.

In chapter 4, my data suggests that serum exosomal miRNA signature can predict a GBM diagnosis. Though again, it is required to have larger longitudinal cohort of GBM patients to assess signature's utility in clinical practice.

In this thesis I focused on serum exosomal miRNAs, whereas there are many other sncRNA subsets present in exosomes that should be investigated in the development of future exosomal sncRNA-based biomarkers. Beyond the sncRNAs, exosomes also carry protein and lipids, which may also have value as biomarkers. Taken together, despite the need for further clinical studies in both MS and GBM, the results are promising enough to warrant investigation of other CNS diseases by the methods developed here.

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Appendix

Table supplementary A-1 MS disease activity phase based on Gd-MRI scans and clinical examinations

De-identifier	Gd(BL)	Clinical (BL)	Gd(6M)	Clinical (6M)	Gd(12M)
1	A	A (34until20days)	A	Q	Q
2	A	Q	A	Q	Q
3	A	Q	A	Q	A
4	A	Q	A	Q	Q
5	A	Q	A	Q	A
6	A	Q	Q	Q	Q
7	A	A (20days_Start)	Q	Q	Q
8	A	Q	Q	Q	Q
9	A	A (46until 16days)	Q	Q	Q
10	A	Q	Q	Q	Q
11	A	A (clinically active in MRI day)	Q	Q	Q
12	A	A (the day of MRI last day of activity)	Q	Q	Q
13	A	Q	Q	Q	A
14	A	Q	Q	Q	Q
15	Q	Q	A	Q	Q
16	Q	Q	A	Q	Q
17	Q	Q	A	Q	A
18	Q	Q	Q	Q	Q
19	Q	Q	Q	Q	Q
20	Q	Q	Q	Q	Q
21	Q	Q	Q	Q	Q
22	Q	Q	Q	Q	Q
23	Q	Q	Q	Q	Q
24	Q	Q	Q	Q	Q
25	Q	Q	Q	Q	Q
26	Q	A (15until8days)	Q	Q	Q

De-identifier	Gd(BL)	Clinical (BL)	Gd(6M)	Clinical (6M)	Gd(12M)
27	Q	Q	Q	Q	Q
28	Q	Q	Q	Q	Q
29	Q	Q	Q	Q	Q