In vivo magnetic resonance imaging for assessing the integrity of the blood-tumour barrier in a mouse model of melanoma brain metastasis

Mariama Henry
The University of Western Ontario

Supervisor
Dr. Paula Foster
The University of Western Ontario

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Mariama Henry 2012

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Medicine and Health Sciences Commons

Recommended Citation

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
IN VIVO MAGNETIC RESONANCE IMAGING FOR ASSESSING THE INTEGRITY OF THE BLOOD-TUMOUR BARRIER IN A MOUSE MODEL OF MELANOMA BRAIN METASTASIS

(Spine title: Assessing BTB integrity of melanoma brain metastases)

(Thesis format: Integrated Article)

by

Mariama Ngozi Henry

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Mariama N Henry 2012
The thesis by

Mariama Ngozi Henry

entitled:

*In vivo* magnetic resonance imaging for assessing the integrity of the blood-tumour barrier in a mouse model of melanoma brain metastasis

is accepted in partial fulfillment of the requirements for the degree of Master of Science
Abstract

Melanoma is the deadliest form of skin cancer. Metastasis to the brain is a life-threatening complication of melanoma in which the clinical incidence is 6-43%. Few animal models exist for melanoma brain metastases, and many are not clinically relevant. MRI was implemented to examine the development of tumors in a clinically relevant model of melanoma brain metastases. Balanced steady-state free precession (b-SSFP) sequence was used to assess total metastasis burden, T1wSE MRI using Gd-DTPA was used to assess blood-tumour barrier (BTB) integrity in vivo and dextran perfusion was used to assess BTB leakiness in situ. This model produced low tumour burden ranging from 5 to 19 metastases at endpoint, many nonenhancing metastases were detected at early time points and there was considerable heterogeneity in permeability of the BTB for melanoma brain metastases. This clinically relevant model can be applied in future studies involving testing efficacy of chemotherapeutic agents.

Keywords

Melanoma, clinically relevant, brain metastasis, balanced steady-state free precession (bSSFP), magnetic resonance imaging (MRI), blood-tumour-barrier (BTB), enhancing, nonenhancing, in vivo, metastasis burden, skin cancer, hematoxylin and eosin, mouse model, gadolinium contrast agent (Gd-DTPA), micron-sized iron oxide (MPIO), 3 Tesla (3T), blood brain barrier (BBB), intracardiac (IC) cell injection, Dextran Texas Red, ex vivo, in situ, cellular MRI.
I would first like to express my sincere gratitude, to the University of Western Ontario, for the opportunity of pursuing my graduate studies in the Medical Biophysics program and, to the Robarts Research Institute for providing the means for me to conduct this unique and interesting research work.

I wish to foremost express my gratitude to my supervisor, mentor, friend and colleague Dr. Paula Foster, who has inspired me to think beyond in regard to research and scholarship. Through her leadership and guidance, I was motivated and excited to create and develop this thesis, with her invaluable help and patience. This was a wonderful experience- working and learning in your lab and for providing financial support for my project.

I would also like to thank the members of my research advisory committee: Dr. Ann Chambers; and Dr. Giles Santyr, who insisted that I become inquisitive and curious during my search, by looking at what other avenues should be explored in this project.

My day-to-day scanning requirements: In order that this research be completed on time; the scanner required constant troubleshooting and replacement of parts for the mouse coils. For this, I am indebted to Dr. Andrew Alejski, Thank you. In addition, I am thankful to Dr. Francisco Martinez for the help in maintaining the chiller, which cools the gradient inserts. Also, I would like to thank Dr. Brian Rutt for construction of the gradient insert; which was heavily used in my MR imaging studies. And Trevor Szekeres for ensuring that the 3T is up in a timely manner, and for timely notification of the system upgrades including 3T downtime.

To my lab associates: Yuhua Chen (for assisting in cutting mouse brain tissue, performing mice perfusions and histological imaging), Catherine McFadden (for assisting in cell culturing of the melanoma cells and the preparation of these cells for intracardiac cell injections), Dr. Emeline Ribot (for assisting with cell injections for the USPIO pilot project and preparation of mice for gas anesthesia before intracardiac cell injections); Carmen Simedrea (for your expertise in intracardiac cell injections - this being the central core of this project); Dr. Laura Gonzalez-Lara (who trained me to use the clinical MRI scanner and for those tricky quizzes you developed to test my knowledge); Dean Percy (for his
expertise in gadolinium scans and for training me to prepare gas anesthesia connections); Phil Gareau (for measuring tumour volumes for comparison with my results); Jonatan Snir (for helping with the inundated animal scans over the summer and helping to troubleshoot alongside with me problems with the mouse RF coils) and Roja Rohani (for aid in the use of statistical software Graphpad Prism® version 5). I am truly grateful for all of your assistance throughout the many phases of my research project.

I would also like to acknowledge, Christiane Mallett (for your help with poster layout and formatting), Vasiliki Economopoulos (for burning files to dvds, and photographing mouse brains), Dr. Amanda Hamilton, Dr. Gabrielle Siegers, and Sherri Couto (for your help with miscellaneous aspects of my project). Your help is greatly appreciated.

To my loving, supportive and compassionate parents, who have inspired me through this journey. You taught me the importance of perseverance, commitment and academic inquisitiveness. You also instilled in me the benefits of gaining invaluable experiences. I would also like to thank my brother, who constantly encouraged me to never give up, but to meet each day with fresh determination in my academic journey. Also, a special thanks to all my extended family and friends for their understanding and kind support.
# Table of Contents

Certificate of Examination.............................................................................................................................. ii  
Abstract.......................................................................................................................................................... iii  
Acknowledgments........................................................................................................................................ iv  
Table of Contents........................................................................................................................................ vi  
List of Tables................................................................................................................................................ x  
List of Figures............................................................................................................................................... xi  
List of Abbreviations.................................................................................................................................... xiv  
List of Symbols............................................................................................................................................. xvii  
List of Appendices....................................................................................................................................... xviii  

## Chapter 1 ................................................................................................................................................. 1  
1 Introduction................................................................................................................................................ 1  
1.1 Skin Cancer........................................................................................................................................... 1  
1.1.1 Melanoma......................................................................................................................................... 1  
1.2 Metastasis................................................................................................................................................ 2  
1.2.1 Mechanisms of Acquiring Blood Supply: Vessel Co-option vs. Angiogenesis................................. 4  
1.3 Melanoma Brain Metastases.................................................................................................................. 5  
1.3.1 Incidence and Metastatic Propensity to the Brain .......................................................................... 5  
1.3.2 Clinical Manifestation....................................................................................................................... 5  
1.3.3 Clinical Treatment and Management............................................................................................... 6  
1.4 The Blood-Brain Barrier (BBB) and Blood-Tumour Barrier (BTB) .................................................... 7  
1.4.1 The Blood-Brain Barrier Structure................................................................................................ 7  
1.4.2 The Blood-Tumour Barrier............................................................................................................. 9  
1.4.3 BBB and Chemotherapeutics........................................................................................................ 10
1.5 Animal Models of Melanoma Brain Metastases.................................................10

1.5.1 Routes of Cell Injections............................................................................10

1.5.2 Melanoma Mouse Models: Genetically Engineered, Xenotransplanted
and Syngeneic....................................................................................................11

1.6 MRI Fundamentals.............................................................................................13

1.6.1 MRI (Magnetic Resonance Imaging): Brief Introduction..........................13

1.7 Physics of MRI...................................................................................................13

1.7.1 Basics of hydrogen atom behaviour.............................................................13

1.7.2 MRI Gradients..............................................................................................16

1.7.3 Excitation and Relaxation..............................................................................16

1.8 Types of MRI Pulse Sequences..........................................................................18

1.8.1 Spin Echo (SE) Pulse Sequence.....................................................................18

1.8.2 Gradient Echo (GRE) Pulse Sequence..........................................................19

1.8.3 Balanced Steady-State Free Precession Pulse Sequence..............................20

1.9 Contrast Mechanisms..........................................................................................22

1.10 Contrast Agents..................................................................................................23

1.10.1 T1 Shortening Agents..................................................................................23

1.10.2 T2 Shortening Agents..................................................................................24

1.11 MRI of Human Brain Metastases......................................................................25

1.11.1 MRI Sequences used in Detection of Human Brain Metastases....................25

1.11.2 MRI Appearance of Melanoma Brain Metastases.......................................26

1.12 MRI of Brain Metastases in Animal Models.....................................................27

1.13 Thesis Overview and Objectives........................................................................29

1.14 References.........................................................................................................30

Chapter 2..................................................................................................................40
In vivo assessment of melanoma brain metastases using longitudinal MRI.

2.1 Introduction

2.2 Materials and Methods

2.2.1 Experimental Groups

2.2.2 Cell culture and Cell Viability Assays

2.2.3 Animal Model

2.2.4 Imaging

2.2.5 Image Analysis

2.2.6 Histology and Microscopy

2.2.7 Statistical Analyses

2.3 Results

2.3.1 Cell Labeling

2.3.2 Imaging

2.3.3 Longitudinal MRI

2.4 Discussion

2.5 References

Chapter 3

3 Study Implications and Future Directions

3.1 Summary of Key Findings

3.1.1 Characterized Model of Melanoma Brain Metastasis using MRI

3.1.2 Longitudinal Magnetic Resonance Imaging Studies: Early Detection of Melanoma Brain Metastases using bSSFP

3.1.3 Longitudinal Magnetic Resonance Imaging Studies: Variability in Enhancement and Patterns of Enhancement

3.1.4 Longitudinal Magnetic Resonance Imaging Studies: Volume, Location and Age of Enhancing and Nonenhancing Brain
Metastases

3.1.5 Histology: Ex vivo Permeability vs. In vivo Enhancement

3.2 Biological Explanations of Metastasis Enhancement

3.2.1 BTB Modulation by VEGF-A

3.2.2 Alteration in Structure of the BBB

3.2.3 Enzymatic Activation and Cell-Cell Communication

3.3 Impact of Key Findings

3.4 Study Limitations

3.5 Future Directions

3.5.1 Histological and Immunohistochemical Characterization of Enhancing and Nonenhancing Melanoma Brain Metastases

3.5.2 Testing Therapeutic Efficacy using Clinically Relevant Model

3.5.3 Translation of the bSSFP Pulse Sequence for Brain Metastasis Detection in the Clinic

3.5.4 Future MRI Studies for Melanoma Brain Metastases

3.6 References

Appendices

Curriculum Vitae
List of Tables

Chapter 1:

Table 1.11.2. Stages of hemorrhage with accompanying changes in T1 and T2 signal intensities (SI) of blood degradation products.................................................................26

Chapter 2:

Table 2.3.1. Number of enhancing melanoma brain metastases over time.........................61
List of Figures

Chapter 1:

Figure 1.1.1. Diagram of skin anatomy and types of skin cancers including melanoma.................................................................2
Figure 1.2.1. The metastatic cascade.................................................................3
Figure 1.3.1. Diagram of human brain meninges..............................................6
Figure 1.4.1. Cells associated with the BBB......................................................8
Figure 1.7.1. Vector components of the net magnetization vector M₀..................15
Figure 1.7.3. T1 recovery curve.........................................................................17
Figure 1.8.1. Diagram of spin echo pulse sequence............................................19
Figure 1.8.3. Diagram of the bSSFP pulse sequence..........................................21
Figure 1.10.1. T1 recovery curve pre and post Gd-DTPA administration.............24

Chapter 2:

Figure 2.3.1. Perls Prussian blue staining of iron-labeled and unlabeled human melanoma A2058 cultured cells.................................................................49
Figure 2.3.2. Cell proliferation assay...............................................................50
Figure 2.3.3. bSSFP image of a mouse brain day 1 post cell injection..................51
Figure 2.3.4. bSSFP images showing the appearance and spatial distribution of melanoma metastases in a representative mouse brain...........................................52
Figure 2.3.5. bSSFP image of a melanoma meningeal metastasis in a mouse brain.................................................................53

Figure 2.3.6. Numbers of melanoma brain metastasis after 50,000 cells injected.................................................................54

Figure 2.3.7. Mean number of meningeal and parenchymal melanoma brain metastasis.................................................................55

Figure 2.3.8. MRI and H&E histology for A2058 melanoma meningeal brain metastases.................................................................56

Figure 2.3.9. MRI and H&E histology for A2058 melanoma parenchymal brain metastases.................................................................57

Figure 2.3.10. bSSFP and T1wSE post Gd-DTPA images of brain metastases.................59

Figure 2.3.11. Heterogeneity in enhancement for A2058 melanoma brain metastases.................................................................60

Figure 2.3.12. bSSFP images show development of a hemorrhagic metastasis and nonhemorrhagic metastases.................................................................63

Figure 2.3.13. Volumes of enhancing and nonenhancing of A2058 melanoma brain metastases at each time point.................................................................64

Figure 2.3.14. Representative bSSFP images comparing volumes for nonenhancing and enhancing melanoma brain metastases from the same mouse.................................65

Figure 2.3.15. Differences in patterns of enhancement over time for A2058 melanoma brain metastases.................................................................66

Figure 2.3.16. Representative T1wSE post Gd-DTPA and bSSFP images of an enhancing metastasis with corresponding Dextran and H&E histology.................................................................67

Figure 2.3.17. Representative T1wSE post Gd-DTPA and bSSFP images of a nonenhancing metastasis with corresponding Dextran and H&E histology.................................................................68
Figure 2.3.18. Representative T1wSE post Gd-DTPA and bSSFP images of an enhancing and nonenhancing metastasis with corresponding H&E histology ........................................69
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>5-FC</td>
<td>5-Fluorocytosine</td>
</tr>
<tr>
<td>ACVS</td>
<td>Animal care and veterinary services</td>
</tr>
<tr>
<td>AIB</td>
<td>Alpha-aminoisobutyric acid</td>
</tr>
<tr>
<td>AJ</td>
<td>Adheren junction</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>b-FGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>bSSFP</td>
<td>Balanced steady-state free precession</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood-tumour barrier</td>
</tr>
<tr>
<td>CD</td>
<td>Cytosine deaminase</td>
</tr>
<tr>
<td>CE</td>
<td>Contrast enhancement</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DCE</td>
<td>Dynamic contrast enhancement</td>
</tr>
<tr>
<td>Deoxy-Hb</td>
<td>Deoxyhemoglobin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FA</td>
<td>Flip angle</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Iron (+3) ion</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FTM</td>
<td>Fotemustine</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>FUS</td>
<td>Focused ultrasound</td>
</tr>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>Gadolinium (+3) ion</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gadopentatic acid or Gadopentetate dimeglumine</td>
</tr>
<tr>
<td>GE</td>
<td>General electric</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetic engineered mouse</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter-1</td>
</tr>
<tr>
<td>GRE</td>
<td>Gradient echo</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>IC</td>
<td>Intracardiac</td>
</tr>
<tr>
<td>ICA</td>
<td>Intracarotid</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>Met-Hb</td>
<td>Methemoglobin</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>MPIO</td>
<td>Micron-sized iron oxide</td>
</tr>
<tr>
<td>NEX</td>
<td>Number of excitations</td>
</tr>
<tr>
<td>NSPCs</td>
<td>Neural stem/Progenitor cells</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>O.C.T</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PBCA</td>
<td>Poly (n-butyl cyanoacrylate)</td>
</tr>
<tr>
<td>PD</td>
<td>Proton density</td>
</tr>
<tr>
<td>PC</td>
<td>Phase cycles</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PPB</td>
<td>Perls Prussian blue</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>rBW</td>
<td>Receiver bandwidth</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Spin echo</td>
</tr>
<tr>
<td>SI</td>
<td>Signal intensity</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SPIO</td>
<td>Superparamagnetic iron oxide</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>STAT-3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>T1w</td>
<td>T1 weighted</td>
</tr>
<tr>
<td>T1wSE</td>
<td>T1 weighted spin echo</td>
</tr>
<tr>
<td>T2w</td>
<td>T2 weighted</td>
</tr>
<tr>
<td>T2*w GRE</td>
<td>T2 star weighted gradient echo</td>
</tr>
<tr>
<td>T2w SE</td>
<td>T2 weighted spin echo</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>USPIO</td>
<td>Ultra small superparamagnetic iron oxide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBRT</td>
<td>Whole brain radiotherapy</td>
</tr>
</tbody>
</table>
# List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_0$</td>
<td>External magnetic field</td>
</tr>
<tr>
<td>$G_s$</td>
<td>Slice selection gradient</td>
</tr>
<tr>
<td>$G_p$</td>
<td>Phase encoding gradient</td>
</tr>
<tr>
<td>$G_f$</td>
<td>Frequency encoding gradient</td>
</tr>
<tr>
<td>$\hbar$</td>
<td>Planck’s constant</td>
</tr>
<tr>
<td>$I$</td>
<td>Spin angular momentum</td>
</tr>
<tr>
<td>$J$</td>
<td>Angular momentum</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>Boltzmann constant</td>
</tr>
<tr>
<td>$M_0$</td>
<td>Net magnetization vector</td>
</tr>
<tr>
<td>$M_z$</td>
<td>Longitudinal magnetization vector</td>
</tr>
<tr>
<td>$M_{xy}$</td>
<td>Transverse magnetization vector</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Proton density</td>
</tr>
<tr>
<td>$T(\degree C)$</td>
<td>Temperature</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Magnetic moment</td>
</tr>
<tr>
<td>$\omega_0$</td>
<td>Larmor frequency</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Gyromagnetic ratio</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix A: Animal use Protocol .........................................................96

Appendix B: Inter and intra observer metastasis volume measurements.........................97

Appendix C: Total metastatic burden ........................................................................100

Appendix D: Total, mean metastasis number and mean volumes for meningeal and parenchymal brain metastases.................................................................101

Appendix E: Melanoma brain metastases hemorrhage.................................................102

Appendix F: Pilot study using USPIO (Molday) for tumour vasculature analysis.............103

Appendix G: Melanin images.......................................................................................105

Appendix H: License and permission usage....................................................................106
“The significant problems we have cannot be solved at the same level of thinking with which we created them”

-Albert Einstein
Chapter 1

1 Introduction

1.1 Skin Cancer

1.1.1 Melanoma

Melanoma is one of three types of skin cancers, it is the second most prevalent cancer to affect young adults ranging in age from 15-29 years of age (1-3). There are three types of skin cancers: basal cell carcinoma, which is the most frequent, squamous cell carcinoma and malignant melanoma. Malignant melanoma is the most dangerous form of skin cancer and it is the leading cause of death among skin cancers (4,5). Melanoma is a malignancy of melanocytes, the pigment producing cells of the skin. Melanocytes produce melanin, which is associated with skin colour but also acts to block ultraviolet (UV) radiation thereby protecting the skin from deoxyribonucleic acid damage (6). Melanomas can appear as black, brown, red, white, pink, purple or blue. Warning signs of melanoma can be detected using the ABCDE method: A=asymmetry in a mole, B=borders are uneven (irregular), C= colour variation in a mole, D= diameter of a mole is larger than 6mm, E=evolving, any changes in a mole such as size, colour, or new behaviour i.e. bleeding, itching (4). Common risk factors associated with melanoma include: exposure to UV radiation (i.e. sun, tanning beds), light-coloured skin, hair and eyes, familial and personal history of skin cancer, sunburns, large number of moles (>50), and atypical moles (6). Figure 1.1.1 shows the skin anatomy, types of skin cancers and the location of melanocytes (7). The skin is divided into two upper layers known as the epidermis and dermis and an underlying third layer known as the hypodermis or subcutis (6). Melanocytes are located in the deepest basal layer of the epidermis (6). Malignant melanoma is deadly since it is capable of metastasizing rapidly to other organs such as liver, lung and lymph nodes producing visceral metastasis, and compared to all primary tumours it has the highest propensity to metastasize to the brain (8,9).
Figure 1.1.1 Diagram of skin anatomy and types of skin cancers including melanoma. The two main layers of the skin include: epidermis and dermis. Each type of skin cancer is derived from three different cell types within the skin epidermis: squamous cells, basal cells and melanocytes. Melanocytes are located at the epidermal and dermal junction of the skin; basal layer of the skin epidermis. Compared to basal and squamous cancers melanoma invades deep into the dermal layer of skin. Source: Reprinted from the MayoClinic.com Image “Where skin cancer develops” (http://www.mayoclinic.com/health/medical/IM02400). ©Mayo Foundation for Medical Education and Research. All rights reserved. With permission for re-usage from Mayo Clinic. (7)

1.2 Metastasis

A common problem associated with cancer is metastasis, whereby cells from a primary tumour spread to other organs in the body (10). Metastasis is the leading cause of death among cancer patients (10). Metastasis occurs by one of two routes: hematogenous or lymphatic dissemination (11,12). Hematogenous dissemination refers to cellular spread via the blood circulatory system whereas lymphatic dissemination refers to cells travelling through the lymphatic vessels. In order for a cancer cell to successfully grow elsewhere in the body it must go through a series of steps that is known as the metastatic cascade (13,14),...
Figures 1.2 shows a diagram of the metastatic cascade. To initiate metastasis, 1) tumour cells must first breakdown the extracellular matrix (ECM), of the surrounding tissue thereby enabling the cells to 2) intravasate into nearby blood or lymphatic vessels. 3) After intravasation, the cells can then travel to distant organ sites. 4) Once the cancer cells arrive at a target organ site, 5) they must arrest by binding to vascular endothelial cells and then 6) extravasate from the blood vessels into the organ parenchyma. Not every cell that escapes from the primary tumour completes all steps of the metastatic process, this may lead to absence in metastasis development, and thus metastasis is an inefficient process. (15-18).

Figure 1.2. The metastatic cascade. (a) The metastatic cascade comprises a series of steps that a tumour cell must undergo to disseminate to a secondary site. (http://www.nature.com/nrc/index.html). Reprinted by permission from MacMillan Publishers Ltd: Nature Reviews Cancer Chambers et al. 2:563-572 copyright © 2002 August Nature Publishing Group. (14)

Metastasis to the brain is the most feared complication of systemic cancer (19) because it is an incurable and a debilitating disease. It is estimated that 20-40% of cancer patients will develop brain metastases (8). Brain metastases are also the most prevalent type of intracranial tumour (8,20). Three common primary cancers that result in brain metastasis are lung cancer,
breast cancer and melanoma (8, 21, 22) all of which result in dismal prognosis as exemplified by short median survivals.

1.2.1 Mechanisms of Acquiring Blood Supply: Vessel Co-option vs. Angiogenesis

Primary tumour cells that successfully extravasate have shown altered mechanisms of target tissue invasion. The two most commonly reported mechanisms of acquiring blood supply are vessel co-option and angiogenesis (23-26). Angiogenesis is the development or formation of new blood vessels (27). This process is mediated by signaling molecules such as vascular endothelial growth factor (VEGF). VEGF is the most common and potent angiogenic factor described in the literature and has been shown to function in the vasodilation of blood vessels, the induction of vascular permeability, and the migration of endothelial cells to form new blood vessel structures (28-31). Previous studies of cancer growth have shown that angiogenesis is a requirement for tumor growth and proliferation (32).

In the brain, however, the large vascular supply often permits metastatic tumour growth without the promotion of angiogenesis (33). Melanoma and breast cancer brain metastases rely on vessel co-option whereby tumour cells use pre-existing blood vessels as their blood supply for growth and proliferation (34). Confocal microscopy analysis of melanoma and breast cancer co-opted vessels revealed that these cells were elongated in a perivascular position or pericyte-like position along cerebral blood vessels (34,35). Kienast et al. used in vivo multiphoton laser scanning microscopy to demonstrate that two melanoma cell lines (MDA-MB-235 and A2058) were capable of invading and proliferating within the brain by co-opting cerebral microvessels. In addition, this study showed that after extravasation into the brain tissue, lung cancer cells (PC14-PE6) proliferated into small clusters that were in close proximity to each another. These clusters eventually fused together to form large lesions where angiogenesis was induced (35).
1.3 Melanoma Brain Metastases

1.3.1 Incidence and Metastatic Propensity to the Brain

The reported clinical incidence of melanoma brain metastasis is 6-43% (36), although autopsy reports have shown higher incidences of 75-90% (22,37). Melanoma is the third most common cancer to cause brain metastasis (19,22) after lung and breast cancer; however, melanoma has the highest propensity to metastasize to the brain compared to all other primary tumours (9,38). Brain metastasis is most often detected within 1 year of primary cancer diagnosis for melanoma (39).

1.3.2 Clinical Manifestation

Clinical cases of melanoma brain metastasis often present with multiple lesions that are multifocal (40). These can occur anywhere in the central nervous system (CNS) and may be associated with leptomeningeal spread (40). Patients can also present with single lesions but these are less common (41,42). Figure 1.3.2 shows the meningeal layers of the human brain. The meninges are membranous linings consisting of the dura, arachnoid and pia (43) that cover the brain and serve to protect it from injury, they also house blood vessels and cerebral spinal fluid (CSF) (44,45). CSF is secreted in the subarachnoid space between the arachnoid and pia mater (45). Other manifestations of melanoma brain metastases include hemorrhage (46), hydrocephalus (abnormal accumulation of CSF in brain ventricles), and mass effects leading to increased fluid pressure (19).

The pattern of seeding for tumour cells in patients with melanoma brain metastasis is as follows: parenchymal (cortex) 49%, leptomeningeal (arachnoid and pia) 22%, and dural 32% (47,48). An autopsy study reviewed by de la Monte et al. showed that the number of CNS cases with metastases due to melanoma was 88% in the cortex, 63% in the meninges and 24% in the dura (49).
Figure 1.3.2. Diagram of human brain meninges. A protective membrane layer known as the meninges covers the brain. There are three meningeal layers: the most outer layer closest to the human skull is known as the dura mater, the second layer is the arachnoid mater, the third layer and is furthest away from the skull is the pia mater. In between the arachnoid and pia mater is the subarachnoid space that contains CSF and blood vessels. Source: Reprinted from the MayoClinic.com Image “Meningitis” (http://www.mayoclinic.com/health/medical/IM00113). ©Mayo Foundation for Medical Education and Research. All rights reserved. With permission for reusage from Mayo Clinic. (44)

The number of melanoma brain metastases detected in humans typically ranges between 1-10 metastases (50,51). Previous studies have shown a correlation between the number of brain metastases and patient survival (19,41,42,52).

1.3.3 Clinical Treatment and Management

Current brain metastases treatments are often palliative and ultimately aim to stabilize neurologic symptoms (8). Potential therapies include radiosurgery, surgery, whole brain radiotherapy (WBRT), corticosteroid treatment (symptomatic treatment to reduce edema) and chemotherapy (8,20,53,54). The number of metastases is an important factor for determining the course of treatment (37,52). Surgery is usually reserved for patients who have a single lesion that is surgically accessible (8). For multiple lesions WBRT is used (9). Despite
treatment efforts, the general prognosis for patients with multiple metastases is poor (52). There is still debate about whether it is the number of metastatic lesions or the lesion volume that impacts patient prognosis the most (52). Melanoma brain metastases are typically radioresistant (8).

1.4 The Blood-Brain Barrier (BBB) and Blood–Tumour Barrier (BTB)

1.4.1 The Blood-Brain Barrier Structure

The BBB functions to protect the brain from foreign materials such as microorganisms and toxic substances (55,56). The core component of the BBB is the endothelium of the brain microcirculation, which is supported by specialized cells such as astrocyte, pericytes, microglia and neurons (55,57). These cells line the endothelial cells of the cerebral vasculature and together they function as a unit to stabilize blood vessel walls and maintain BBB integrity (56). Figure 1.4.1 shows the structure and cell composition of the BBB.
Figure 1.4.1 Cells associated with the BBB. This figure illustrates the specialized cells associated with the BBB, which include pericyte, astrocytic foot processes, neurons, and microglia. These cells are closely associated with the cerebral endothelial cells and together form a structural barrier. Tight junctions connect the cerebral endothelial cells. BL1 and BL2 are two different extracellular matrices of the basal lamina (BL); BL1 represents a distinctive extracellular matrix in association with endothelial cells and pericytes, BL2 represents a different extracellular matrix in association with astrocytic foot processes. Axonal terminals of neurons (in close proximity to smooth muscles) contain vasoactive neurotransmitters and peptides that control cerebral blood. Reprinted from Neurobiology of Disease, volume 37, N Joan Abbott, Adjanie A.K. Patabendige, Diana E.M. Dolman, Siti R Yusof, David J.Begley, Structure and function of the blood-brain barrier, pages 13-25, Copyright © 2009, with permission from Elsevier. (56)

Other structural components of the BBB include tight junctions (TJ) and adheren junctions (AJ). These junctions are responsible for controlling the paracellular permeability of the BBB endothelium (58,59). The TJ, function in adjoining adjacent endothelial cells forming a seal (55). AJ are responsible for the development and maintenance of TJ and also form connections between endothelial cells (55, 58).

The BBB is a selective barrier. Generally small molecules <400-600 daltons (Da), neutral and lipophilic, are more likely to permeate the BBB (55,60). Molecules that are essential for brain function that can permeate across the BBB include ions, blood gases \( O_2 \), and \( CO_2 \) that
enter via passive permeability (59); glucose and amino acids which enter via solute transporters; proteins and peptides which enter via transcytosis through receptor mediated mechanism (59).

Disruption of the BBB is often indicative of the presence a pathological condition (56), thus changes in permeability can be seen as an early indicator of an abnormality within the brain. Dextran is commonly used as an ex vivo tracer to monitor BBB integrity, it ranges in size from 3 kilo Dalton (kDa) - 70kDa (61). It is also important to note that permeability is a dynamic process that coincides with altered demands for nutrients, protection from foreign substances, local repair processes and other requirements that might be necessary for proper function (56). Although the BBB acts to protect the brain from foreign agents that are detrimental to brain function (62), some metastatic cells are able to penetrate the BBB and invade the brain parenchyma (47).

1.4.2 The Blood-Tumour Barrier

The BTB refers to the interface between tumours and tumour-associated blood vessels. Blood vessels in regions of the BTB can have altered barrier integrity (63) possibly due to macromolecules secreted by tumour cells such as VEGF-A and basic fibroblast growth factor (b-FGF) (64). Tumour-associated blood vessels have been shown to exhibit features more similar to systemic blood vessels rather than their cerebral counterparts (63). In addition, tumour-associated blood vessels are abnormal in that they exhibit convoluted patterns of branching, tortuosity (meaning that they twist and bend unusually), and an altered propensity for leakage (65) compared to the blood vessels of the BBB in the surrounding brain parenchyma tissue (66). Blood vessels of the BTB have also been shown to be co-opted (35) and may also demonstrate sprouting angiogenesis (67).
1.4.3 BBB and Chemotherapeutics

Chemotherapeutic agents range in size from 600 – 1400 Da (63). Most chemotherapeutics are not able to cross the BBB and the BTB and subsequently have had limited efficacy in the treatment of brain metastases. In metastatic melanoma, both systemic and brain metastases typically exhibit an overall poor response rate to systemic chemotherapy (68). Some chemotherapeutics agents, such as temozolomize (TMZ) and nitrosourea fotemustine (FTM), have been shown to cross the BBB and treat melanoma brain metastases. However, the use of these drugs as single agents showed a modest tumor response rate in the case of nitrosourea FTM and a low tumor response rate for TMZ (69-71). TMZ and nitrosourea FTM administered in combination with other therapeutic agents, e.g. WBRT or thalidomide (anti-angiogenesis agent), has also shown limited success; with a median survival rate between 2-6 months (21,71-73). There is an urgent need for new, more effective therapies against brain metastasis (39).

1.5 Animal Models of Melanoma Brain Metastases

1.5.1 Routes of Cell Injections

Animal models of melanoma brain metastasis that mimic hematogenous spread can be derived by intracardiac (IC) or intracarotid (ICA) cell injection. Less commonly used cell injection routes include: intravenous (IV) (injection of cells into a vein), intracerebral/intracranial (direct implantation of cell into the cerebral cortex) and intrathecal (injection of cells into the subarachnoid space) (74). The intracarotid cell injection method has been the most extensively used for producing blood-borne experimental brain metastases, however, a few previous studies have successfully used the intracardiac cell injection method for the production of brain metastases (34,61,75-81). Although intravenous injections are based on cells injected into the venous circulation they are not suitable for brain metastasis production since the first capillary bed encountered by cells is the lungs. Intracerebral/intracranial and intrathecal cell injection routes are not representative of hematogenous spread since tumour cells are not injected into the circulatory system.
IC injections involve injecting cells into the left ventricle of the beating animal heart. An advantage of using the IC method is that it is relatively simple in terms of cell delivery (74). Meanwhile, ICA involves injecting cells into the internal carotid artery. This method of cell injection eliminates cell distribution to other organ sites however it requires extensive skills in microsurgery (74) and typically only one side of the brain shows presence of metastases.

1.5.2 Melanoma Mouse Models: Genetically Engineered, Xenotransplanted, and Syngeneic

Animal models of melanoma brain metastasis commonly use human cells that are transplanted into athymic nude (nu/nu) mice. Tumours are able to grow in nude mice because they lack a thymus thereby making them incapable of producing mature T cells that are involved in the adaptive immune system and are responsible for the recognition of foreign antigens. Human tumours heterotransplanted into immunodeficient mice are also able to retain their morphologic and biochemical characteristics (82,83).

Most models of brain metastases can be classified as either spontaneous or experimental (84). Three types of mouse models for melanoma have been used experimentally: genetically engineered models (GEM) are a type of spontaneous model, xenotransplantation models and syngeneic transplantation model (85) are two different types of experimental metastasis models. Xenotransplantation refers to orthotopic implantation of cells from one species into another (i.e. human primary or secondary tissue into a mouse model) (13) whereas syngeneic transplantation refers to implantation of cells from the same species origin. Each of these models serves a different purpose depending on the type of experimental question being asked. For instance, GEM such as BRAFV600E Cdkn2a +/- background (86) are useful for studying the early metastatic progression such as the initiation of tumorigenesis; genetic changes in melanocytes turns them into malignant melanoma cells. A disadvantage in this type of model is that GEM have shown limited capability of producing metastasis (85,87).
Xenotransplantation models are useful for looking at metastatic behaviour. An example of a xenotransplantation model is the A375-SM metastatic melanoma lymph node cell line injected into nude mice (47,74,82,88). Syngeneic models are useful for monitoring immune reactions in response to allograft cells that were injected e.g. a common syngeneic model used in melanoma research is the injection of B16 melanoma cells into C57BL/6 mice (74,89).

Most experimental metastasis models inject tumour cells directly into the circulation (84), thereby bypassing the early stages of the metastatic cascade (13). In this method emphasis is placed on the later stages of metastasis, which includes cell arrest in capillary beds, extravasation, invasion, angiogenesis or vessel co-option and proliferation. Spontaneous metastatic models encompass the entire metastatic cascade from early stages (primary site) to late stages (secondary site) (13). The site of inoculation is where a primary tumour is established and the animals are monitored for the formation of visceral metastases (84). A few spontaneous models of melanoma have been reported that demonstrate metastasis to the brain (90,91). For instance Alterman et al. have shown that that B16 clones G3.5 and G3.12 were capable of producing spontaneous metastasis to the brain and Cruz Munoz et al. have demonstrated that the metastatic melanoma cell line 113/6-4L derived from the WM239A human melanoma parental cell line also showed spontaneous metastasis to the brain. Spontaneous models of melanoma brain metastasis are rare and less reproducible; few cell lines have been reported that are capable of spontaneously metastasizing to the CNS from a primary tumour (92). For this reason experimental metastasis models are more commonly used compared to spontaneous models to study experimental brain metastasis.


1.6 MRI Fundamentals

1.6.1 MRI (Magnetic Resonance Imaging): Brief Introduction

MRI is a widely used noninvasive imaging modality. Advantages of MRI include excellent soft-tissue contrast, high sensitivity, high-resolution and no ionizing radiation source (93). Magnetism or magnetic fields is the core foundation for imaging in MRI (93). The principle of MRI stems from the inherent properties of protons or hydrogen atoms (1H nuclei) (93). The human body is composed of 75% to 80% water and fat (93) providing a large pool of protons that enables MR imaging.

1.7 Physics of MRI

1.7.1 Basics of hydrogen atom behaviour

The signal acquired from MRI is based on nuclear magnetic resonance (NMR). The nucleus of hydrogen atoms consists of one positively charged proton. The hydrogen atom possesses an odd number of protons, thus it will exhibit nuclear spin, which is a quantum mechanical property as well as spin angular momentum. A moving charged particle (e.g. a proton) can induce a magnetic field. The larger the magnitude of the charge or speed, the greater the magnetic field induced. The magnetic field produced is known as the magnetic moment (\( \mu \)), which is directly proportional to angular momentum (93) (refer to equation 1.7a). The magnetic moment is a vector quantity (94).

\[
\mu = \gamma J = \gamma hI \quad (93) \quad \text{(Equation 1.7a)}
\]

Where \( \mu \) is the magnetic moment, \( \gamma \) is the gyromagnetic ratio, \( J \) is the angular momentum, \( h \) is Planck’s constant divided by \( 2\pi \) and \( I \) is the spin angular momentum number equaled to \( \frac{1}{2} \) for protons (93).
In the absence of an external magnetic field, hydrogen atoms spin randomly where the vector sum of the magnetic moments is zero. In the presence of an external magnetic field, the spins of protons are described as being quantized (distributed amongst different energy levels), in the case of the hydrogen atom two energy states exist. When protons are placed in an external magnetic field some spins are slightly aligned with the magnetic field (parallel direction); lower energy state and some spins are slightly aligned in the opposite direction (anti-parallel direction) of the magnetic field; higher energy state. Parallel and anti-parallel spins will cancel out but a small subset or excess of spins will be aligned with the magnetic field producing a net magnetization vector. Figure 1.7.1 shows the vector components of the net magnetization vector, which are $M_z$ and $M_{xy}$ (96).

As you increase in magnetic field strength, the magnitude in energy difference increases for energy states. More spins will be slightly aligned with the magnetic field. In reality the spins of protons switch back and forth from the two energy states and the ratio of protons that are parallel and anti-parallel will depend on the magnetic field strength and temperature that is inversely proportional to the ratio of parallel and anti-parallel spins of protons (refer to equation 1.7b).

\[
M_0 = \frac{\rho \gamma^2 \hbar^2 B_0}{4 \kappa B T (°C)} \quad (93) \quad \text{(Equation 1.7b)}
\]

Where $M_0$ is the net magnetization, $\rho$ is the proton density, $B_0$ is the magnetic field strength, $\kappa$ is the Boltzmann constant $1.38 \times 10^{-23} \text{JK}^{-1}$, $T$ is the temperature (body temperature $37°\text{C}$) (93). By conducting experimental measurements (e.g. collecting MRI signal) we are able to know the probability of distribution of spins among the energy levels.
Figure 1.7.1 Vector components of the net magnetization vector $M_0$. $M_0$ is the net magnetization vector which is the sum of the $M_{xy}$ and $M_z$ vector components. $M_{xy}$ is the vector component in the $xy$ transverse plane and $M_z$ is the vector component along the $Z$ axis longitudinal plane.

In the presence of a magnetic field, the protons resonate, or precess, at a given frequency, this frequency is known as the Larmor frequency and is defined by the following equation:

$$\omega_0 = B_0 \times \gamma \ (91) \quad \text{(Equation 1.7c)}$$

Where $\omega_0$ is the Larmor frequency, $B_0$ is the magnetic field strength and $\gamma$ is the gyromagnetic ratio ($\gamma$), which is a constant value for a particular nucleus. The units of $\gamma$ are megahertz (MHz) per Tesla (T). The Larmor frequency at 3T for proton nuclei is 127.71 MHz, where $B_0 = 3T$ and the gyromagnetic ratio for protons is $\left(\frac{\gamma}{2\pi}\right) = 42.57 \text{ MHz T}^{-1}$. 
1.7.2 MRI Gradients

Gradient coils are used in MRI to create short-term spatial variations in the magnetic field strength (93). Gradient coils create linear gradients along all three axes x, y, and z (G_x, G_y and G_z) (93) during imaging. The Larmor frequency of protons changes along the linear gradients because each proton senses a different magnetic field strength (93,96).

Slice selection gradients (G_s) select a region of the sample to be imaged. This is the target slice for the radiofrequency (RF) pulse (composed of an electromagnetic wave) application (93,96). Phase encode gradients (G_p) assign a different phase (position/direction) to protons in the selected image slice (93). Frequency encoding gradients (G_f) assign a different frequency to protons in the selected image slice in one direction. The information of each frequency and phase encoding from the image is stored in an image matrix consisting of rows and columns in which each box in the matrix is known as a voxel or voxel volume. Each voxel has a unique frequency and phase code. The voxels are assigned a unique number and are stored as raw data in k-space (93). A mathematical program known as Fourier transform is used to convert the raw data stored in k-space into image space where the image is created (93).

1.7.3 Excitation and Relaxation

Protons are excited through absorption of energy from an RF pulse that corresponds to the Larmor frequency, causing tipping of protons into the transverse (xy) plane (93,94). After the RF pulse is removed, protons relax back to their resting state (lower energy) by three different mechanisms (93,94): T1 recovery, T2 relaxation and T2* relaxation (93,94).

T1 recovery, also known as spin-lattice relaxation or longitudinal relaxation (93,94), is where protons release energy into the surrounding lattice or tissue environment in order to return to their resting state (93, 96). During T1 recovery the M_z vector component grows in size along the z-axis as it returns back to equilibrium. This relaxation is represented by an exponential
curve described as a T1 recovery curve where equilibrium of protons is reached when 63% of the vector has been recovered, this is the T1 value or time. (93,94). Each tissue has a different T1. Figure 1.7.3 shows a diagram illustrating T1 recovery of protons.

![T1 Recovery Curve](image)

**Figure 1.7.3 T1 recovery curve.** When protons relax back to equilibrium, T1 is defined as the time reached where 63% of the original signal has recovered.

T2 relaxation, also known as spin-spin relaxation or transverse relaxation is defined by protons sensing different magnetic field strengths caused by the dephasing interactions from neighbouring protons (93,96). This results in protons precessing at various frequencies and eventually become out of phase. During this dephasing, the magnetization vector $M_{xy}$ decreases in magnitude resulting in signal decay or T2 decay (93,94). T2 decay is represented by an exponential decay curve. The time it takes for the signal to reach 36% of the original signal is referred to as T2 (93,94). Each tissue has a unique T2 value (94). Aside from inherent dephasing properties, protons are also sensitive to magnetic field inhomogeneities that can be caused by air and metal objects. In the presence of random T2 effects (loss of phase) as well as field inhomogeneities (non-homogenous magnetic field strength), protons
decay more rapidly. This is referred to as T2* decay. This phenomenon is represented by an exponential decay that represents signal loss.

1.8 Types of MRI Pulse Sequences

In MRI two types of fundamental pulse sequences are used: spin echo (SE) and gradient echo (GRE). Various MRI pulse sequences are classified in either the SE or GRE pulse sequence families (93). The pulse sequences utilized in this thesis are a T1 weighted SE (T1wSE) sequence and the balanced steady-state free precession (bSSFP) sequence, which is a GRE pulse sequence.

1.8.1 Spin Echo (SE) Pulse Sequence

The SE sequence uses an excitation pulse of 90° to tip protons into the transverse plane. At TE/2, a 180° refocusing pulse is used to bring protons back into phase, and at a time TE the in-phase protons produce an echo (signal acquired) (94). Three types of gradients are used during image acquisition for SE pulse sequence: The slice selection gradient is turned on when the excitation pulse is released, the phase encode gradient encodes different phases for protons. The frequency or readout gradient is turned on at the time the echo is acquired. Figure 1.8.1 shows the diagram of a spin echo pulse sequence.
1.8.1 Diagram of spin echo pulse sequence. This pulse sequence has a 90° excitation pulse and a 180° refocusing pulse. After applying the refocusing pulse, at a time TE an echo (signal) is acquired. $G_{SS} =$ slice selection gradient, $G_{PE} =$ phase encoding gradient, $G_{FE} =$ frequency encoding gradient, TR repetition time, TE echo time. Modified from (93).

1.8.2 Gradient Echo (GRE) Pulse Sequence

GRE sequences use flip angles that are less than 90°. Only one RF pulse is used, and instead of using a refocusing pulse, out of phase protons are rephased through the application of a negative gradient opposite in direction. When protons are in-phase an echo is generated (93,94). Unlike SE sequences, GRE sequences are sensitive to inhomogeneities. GRE sequences are faster and thus shorter scan times are produced (93).
1.8.3 Balanced Steady-State Free Precession Pulse Sequence (b-SSFP):

The bSSFP pulse sequence is a member of the GRE pulse sequence family (93). In a bSSFP pulse sequence the gradients are balanced to produce a signal that eventually remains in ‘steady state’. The net magnetization vector is in steady state because in between each TR interval the balanced gradients refocus the signal to generate a single magnetization vector. During each TR interval T1 and T2 relaxation is occurring but do not recover or decay fully due to rephasing caused by balanced gradients, the resulting magnetization vector is used in the next TR period and the process is repeated multiple times (97).

The contrast in bSSFP is related to T2/T1 (97). Figure 1.8.3 shows the bSSFP pulse sequence diagram. bSSFP is regarded as a pulse sequence that produces the highest signal-to-noise ratio (SNR) per unit time (98) because it is able to maintain a steady and refocused signal at every TR cycle. An advantage of using bSSFP is that high resolution images can be generated while maintaining a high SNR in reasonable scan times.
Applications of bSSFP

Heart cine imaging is the major clinical application of bSSFP (97). bSSFP has also been used in cardiac imaging and angiography for delineation of small structures (97). bSSFP has also more recently been used to generate very high resolution images useful for the differentiation between pineal cysts and pineal tumours (99).

bSSFP has been used in our research group for several studies involving cellular MRI whereby cells are labeled and tracked post cell injection. bSSFP is sensitive to iron due to inhomogeneities produced by the iron agent. bSSFP has been used to track and detect iron labeled cells in various disease models such as prostate cancer, spinal cord injury, brain metastasis (breast cancer) and brain cancer (glioma) detection, development and progression,
dendritic cells in cancer immunotherapy, melanoma metastasis to lymph nodes, and multiple sclerosis (80,81,100-106).

1.9 Contrast Mechanisms

Two key imaging parameters important for image contrast are repetition time (TR) and echo time (TE) (96). The repetition time is defined as the time between excitations (94). The echo time is the time from when the RF pulse is first applied to when the MRI signal is detected (95). By adjusting the TR and TE, three common image contrasts can be generated in MRI (96): T1 weighted (T1w), T2 weighted (T2w) and Proton Density weighted (PD) contrasts.

In T1w images, tissues with a long T1 time (e.g. fluids) appear dark and tissues with short T1 (e.g. fat) appear bright. T1w images are typically used to look at the anatomy and are known as anatomy scans (93). For SE sequences T1w images are produced using a short TR and TE. Typically a flip angle (FA) greater than 50° and short TE times are used to generate T1w contrasts for GRE sequences (93).

In T2w images tissues with a long T2 (e.g. fluids) appear bright and tissues with a short T2 appear dark. Clinically T2w images are referred to as pathology scans since most pathologies have associated edema, which appears very bright (93). T2w images are produced by using long TR and TE for SE sequences and using FA smaller than 40° and long TE for GRE sequences (93). PD images are based on the density of protons of water and fat in a volume of tissue (94). PD images are produced using a long TR and a short TE for SE sequences and using FA smaller than 40° and short TE for GRE sequences (93).

In bSSFP images tissues show T2/T1 contrast, e.g. grey and white matter show low soft-tissue contrast because the ratios of T2 and T1 are the same. Fats and water produce high signal intensities (SI) in bSSFP. In the case of hemorrhage, shortening of T2 and T1 produce
isointense and hyperintense signals. Shortening of T2 and T1 (the more predominant effect due to the presence of extracellular methemoglobin (Met-Hb)) produces hyperintense signals, and shortening of T2 (the more predominant effect due to the presence of superparamagnetic particles such as hemosiderin) and T1 produces hypointense signals.

## 1.10 Contrast Agents

### 1.10.1 T1 Shortening Agents

Gadolinium contrast agents are paramagnetic (93,94) and referred to as T1 shortening agents since they shorten T1 of tissues and post-contrast tissues appear bright (hyperintense) on T1w images (93). Figure 1.10.1 shows T1 shortening (increase in T1 signal; hyperintense) of T1 after administration of gadolinium contrast agent. In the case of brain metastases with disrupted BTB, Gd-DTPA is able to leak into the metastases and cause T1 shortening of neighbouring protons within the metastasis. These contrast agents are used extensively to monitor or measure the BBB and/or BTB integrity (63). The most widely used gadolinium contrast agent in MRI clinical imaging is gadolinium-diethylenetriaminepentaacetic acid or gadopentetate dimeglumine (Gd-DTPA) also known as Magnevist®. Gd-DTPA is composed of a metal ion Gd$^{3+}$ that is chelated by the pentaacetic acid ring. This agent has 7 unpaired electrons (94), which causes gadolinium to be paramagnetic. Gadolinium agents used to monitor BBB integrity range in size from 590 to 950 Da. (63)
**Figure 1.10.1 T1 recovery curve pre and post gadolinium administration.** Before the administration of gadolinium contrast agent, T1 is longer (dark; hypointense signal) (light blue curve). After the administration of gadolinium contrast agent T1 is shortened (bright; hyperintense signal) (dark blue curve).

1.10.2 T2 Shortening Agents

The most common T2 shortening agents are based on iron oxide. Iron-based contrast agents cause protons or hydrogen atoms from water molecules in neighboring tissues to undergo spin dephasing. The resulting spin dephasing from protons causes hypointense regions to be visualized on MRI images. This causes protons to spin at different frequencies, eventually resulting in rapid signal loss (hypointensity). The signal loss results in production of an image artifact known as a ‘blooming artifact’. This is visualized in the MRI image as small
black dots termed signal voids (80). The size of the blooming artifact is much larger than the cell itself, which makes it easy to visualize (98).

Iron contrast agents were first used in liver imaging studies involving tumor detection (93,107). Iron contrast agents are engulfed by liver Kupffer cells (specialized macrophages) and these labeled liver cells cause a decrease in T2 (hypointense) of the normal liver tissue. Liver cancerous lesions are visible as areas of increased signal intensity (hyperintense) since they are devoid of Kupffer cells (93,107).

Today, the field of ‘cellular MRI’ is largely based on the use of iron contrast agents to label cells for tracking using MRI. There are many types of iron contrast agents such as ultra small paramagnetic iron oxide (USPIO), super paramagnetic iron oxide (SPIO) and micron-sized paramagnetic iron oxide (MPIO). They are usually categorized according to their hydrodynamic diameter and size ranging from nanometers to micrometers (108-110). Nearly all cell types can be labeled with sufficient amounts of iron to allow their detection by MRI. Various studies have shown that the incorporation of iron into cells did not interfere with normal cellular functions such as proliferation and migration and did not impact viability (80).

1.11 MRI of Human Brain Metastases

1.11.1 MRI Sequences used in Detection of Human Brain Metastases

MRI sequences typically used for detection of human brain metastases include pre- and post-Gd-DTPA T1wSE (111), T2wSE sequence and T2*w GRE. T1wSE with Gd-DTPA is used for assessing BBB integrity (112), T2wSE is used for detection of the presence of edema. The T2*w GRE pulse sequence is used for detection of susceptibility effects caused by degradation products and melanin (111).
1.11.2 MRI Appearance of Melanoma Brain Metastases

Melanoma brain metastases have variable MRI patterns, mostly attributed to the presence of hemorrhage and melanin (113). MRI of hemorrhage itself has many different appearances. Intracranial hemorrhages change over time as they go through acute to chronic phases where the hemoglobin (Hb) molecule undergoes changes (114,115,116,117). The evolution of blood degradation products at different stages of hemorrhage is shown in Table 1.11.2. This table shows the stages of hemorrhage and associated MRI signal intensities (SI) (hyperintense, isointense, hypointense) depending on the degradation product present. The MRI appearance of blood degradation products due to hemorrhages also depends on the MRI field strength and the MRI sequence employed (114).

<table>
<thead>
<tr>
<th>Stages of hemorrhage</th>
<th>Duration</th>
<th>Blood degradation products of Hb</th>
<th>T1w SI</th>
<th>T2w SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperacute hemorrhage</td>
<td>&lt; 12h</td>
<td>RBC-oxyhemoglobin</td>
<td>↔ or ↓</td>
<td>↑</td>
</tr>
<tr>
<td>Acute hemorrhage</td>
<td>Hours to days</td>
<td>RBC-deoxyhemoglobin</td>
<td>↔ or ↓</td>
<td>↓</td>
</tr>
<tr>
<td>Early subacute hemorrhage</td>
<td>Few days</td>
<td>RBC-methemoglobin</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Late subacute hemorrhage</td>
<td>4-7 days to 1 month</td>
<td>Extracellular-methemoglobin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Chronic hemorrhage</td>
<td>Weeks to years</td>
<td>Hemosiderin and Ferritin</td>
<td>↔ or ↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Table 1.11.2. Stages of hemorrhage with accompanying changes in T1 and T2 signal intensities (SI) of blood degradation products. RBC= Red Blood Cell, Hb= Hemoglobin ↓= marked decrease in signal intensity, ↑=marked increase in signal intensity, ↔ = isointense signal intensity, ↓= decrease in signal intensity, ↑= increase in signal intensity.
Melanin has been shown to act as a T1 and T2 shortening agent (118). Amelanotic (the absence of melanin pigmentation) melanomas tend to be hypointense or isointense relative to surrounding brain tissue in T1wSE images and isointense to hyperintense in T2wSE images. Melanotic melanomas tend to be hyperintense in T1wSE images and hypointense in T2wSE images (46, 113). Some studies have shown that melanin impacts the T1 relaxation time much more than T2 times. Gaviani et al. demonstrated a more profound T1 shortening effect from melanin since they observed a direct correlation between melanin content and T1 hyperintensity. However they did not observe a correlation between melanin and susceptibility effect (111). Similarly, Prekumar et al. observed no correlation between melanin and T2 shortening in visceral melanoma metastasis (119).

### 1.12 MRI of Brain Metastases in Animal Models

MRI, has been used in various studies involving brain metastasis, whereby the implementation of VEGF-A isoforms in a melanoma cell line Mel57 has been used in Gadolinium contrast enhancement (CE) MRI for detection of brain lesions and to monitor their permeability behaviour based on Gd-DTPA uptake. USPIO CE was also used to characterize the vasculature of primary brain tumours (U87 and E34) and metastatic melanoma brain lesions that were transfected with VEGF-A isoforms (67,120,121). MRI techniques such as dynamic contrast enhancement (DCE-MRI) and diffusion weighted MRI (DWI) have been used in animal studies of breast cancer brain metastasis (MDA-MB-231BR) to acquire information about metastasis, permeability and cellularity in the brain and bone microenvironment (122). Therapeutic treatments of brain metastases have also used MRI to evaluate efficacy of the therapeutic drug agent. Antiangiogenic therapy ZA6474 (anti-VEGF) caused angiogenic growth of the melanoma transfected cell line Mel57-VEGF-A to change to the vessel co-option phenotype (123). Gril et al. used MRI to monitor treatment response of the HER2 breast cancer brain metastasis variant (MCF7-HER2-BR3) toward Pazopanib. A 55% reduction in tumour volume was observed with administration of Pazopanib compared to the vehicle control (no drug) (124). From prostate cancer, brain metastasis studies using MRI JuanYin et al. showed that treatment response towards anti-VEGF (AZD2171) administration resulted in two different functional responses of prostate
cancer brain metastasis: tumour cells were able to extravasate into the brain tissue and a heterogenous response in terms of therapy using anti-VEGF for individual brain lesions was also evident (125). In a different study for prostate cancer brain metastasis using AZD2171/Cedarinib, Yin et al. also demonstrated that DU145 prostate cancer brain and bone metastasis growth was inhibited and mice bearing tumours showed a decrease in morbidity and mortality (126).

Multimodality imaging has also been incorporated in animal studies using MRI and bioluminescence imaging (BLI). Song et al. utilized the brain-seeking human breast cancer cell line MDA-MB-231BR in MRI and BLI of rats, to monitor development of breast cancer brain metastasis as well as disease progression in other anatomical sites/structures such as the bone (127). MRI longitudinal studies involving animals has proven to be useful for monitoring tumour development and progression. Perera et al. demonstrated the utility of using MRI to monitor temporal and spatial development of experimental brain metastases in the mouse brain for two metastatic breast cancer cell lines: MDA-MB-231BR and MDA-MB-231BR HER2 (103). Cell tracking of tumour development after the extravasation was demonstrated by Heyn et al. in which the fate of single-cells during the metastatic process of breast cancer brain metastasis was monitored longitudinally (80). MRI longitudinal studies involving permeability of breast cancer brain metastasis is a fairly new area of brain metastasis research. Percy et al. had used a unique imaging protocol to investigate the metastatic burden and alterations of BTB of breast cancer brain metastasis longitudinally in mice (81). It was observed that small metastases were detected with bSSFP at the early time points but they did not enhance with T1wSE using Gd-DTPA. In this model it was also shown that altered BTB and total metastatic burden increased over time.

Although MRI has been used to investigate models of melanoma brain metastasis, longitudinal studies have not been implemented for studying alterations of the BTB in a clinically relevant model of melanoma brain metastasis.
1.13 Thesis Overview and Objectives

The overall goal of this project was to use in vivo MRI to monitor the development of melanoma brain metastases and the integrity of the BTB in a clinically relevant mouse model.

The specific aims of this thesis were as follows:

1) To characterize a clinically relevant model of melanoma brain metastases.

2) To determine the percentage of melanoma brain metastases permeable to Gadolinium contrast.

3) To understand when and why some brain metastases become enhanced while others do not.

4) To examine histological differences between enhancing and nonenhancing melanoma brain metastases.

Chapter 2 discusses the use of a clinically relevant animal model implemented in longitudinal MRI studies monitoring changes in the BTB permeability of melanoma brain metastases over time. Finally, Chapter 3 summarizes the key experimental findings and significance of this study in relation to preclinical translational models as well as implications for clinical disease. Lastly, study limitations and suggested future directions to further improve the melanoma brain metastasis model are discussed.
1.14 References


44. Mayo Clinic-Skin Cancer (Diseases & Conditions)


Chapter 2

2 In vivo assessment of melanoma brain metastases using longitudinal MRI

Work presented in this chapter was done in collaboration with Dr. Ann Chambers, Dr. Paul Lockman and Dr. Patricia Steeg. Catherine McFadden performed cell labeling with MPIO, cell counting for cell injections and A2058 proliferation assay days 0 and 2, Perls Prussian blue staining and cell culturing. Carmen Simedrea performed intracardiac cell injections. Dextran perfusions, perfusions using 4% PFA, cutting of frozen as well as paraffin embedded mouse brain tissue and H&E images of whole mouse brain was performed by Yuhua Chen (Foster group laboratory manager). Dr. Emeline Ribot performed Molday USPIO tail vein injections and Christiane Mallett imaged at day 14 pre and post Molday USPIO tail vein injection. Custom-built MRI hardware, such as the gradient coil insert and the mouse solenoid radiofrequency head coil, was designed and constructed by Dr. Brian Rutt’s laboratory. The remaining procedures including MR scanning, mouse anesthesia, intraperitoneal (IP) injections of gadolinium contrast, monitoring health status and weekly weighing of mice, data analysis, tissue dissection into different brain regions (frontal, midbrain, and hindbrain) in preparation for embedding, tissue embedding of mice brains in O.C.T medium and paraffin wax, H&E staining of frozen and paraffin mouse brain tissue sections, microscopic analysis of H&E slides and fluorescence microscopy, cell counts for A2058 cell culture proliferation assay days 5 and 7 and imaging mouse brain at day 31 pre and post Molday USPIO tail vein injection were performed by Mariama Henry.
2.1 Introduction

Malignant melanoma is the most dangerous form of skin cancer (1). The incidence of malignant melanoma continues to rise, especially among young adults (1). A common complication for patients with malignant melanoma is metastasis to the brain (2). Patients with metastatic melanoma have a 40% to 60% risk of developing brain metastases (3). Moreover, autopsy results have shown that metastases in 70-90% of brains from patients that died with stage IV melanoma (3,4), metastasize to the brain compared to other primary tumours, such as lung and breast cancer (3,5). The prognosis for patients with melanoma brain metastasis remains dismal with a median survival of 1-2 months without treatment (6-8), 4-6 months with treatment (6,9) and a 1-year survival rate in less than 13% of patients (10). Chemotherapeutics are not able to cross the blood-brain barrier (BBB) and consequently have limited efficacy in the treatment of brain metastases (5,11).

The local BBB associated with brain tumours is referred to as the blood-tumour barrier (BTB) (12-15). Several studies have now described significant heterogeneity in the permeability of the BTB associated with individual brain metastases (16,17). Lockman et al. showed that the BTB of breast cancer brain metastases in mice exhibited significant heterogeneity in permeability to dextran and that permeability was unrelated to the size or morphology of metastases (16). Zhang et al. examined brain metastases produced by 8 human cancer cell lines and reported that permeability to sodium fluorescein varied depending on tumour type and was related to tumour morphology and size. Small compact metastases were not permeable until they reached 0.2mm² and diffuse metastases were permeable only when they coalesced to form a large mass (18). These were both ex vivo experiments conducted at specific endpoints and therefore provide only a snapshot of the permeability status of a metastasis.

Magnetic resonance imaging (MRI) has also been used to evaluate BBB integrity in vivo (18-28). Gadolinium contrast agents such as Gadopentatic acid (Gd-DTPA) are used routinely in MRI to demonstrate BBB breakdown (17,29-33). Gadolinium cannot cross an
intact BBB, therefore extravascular enhancement in MR images after administration of Gd-DTPA indicates a leaky BBB, or increased permeability (34). Percy et al. used Gd-DTPA enhanced MRI to evaluate BBB permeability and tumour burden in a mouse model of breast cancer metastasis to the brain (17). This paper showed significant heterogeneity in the permeability of these brain metastases with increasing numbers of permeable tumours over time. This study also revealed that small metastases could be detected using the balanced steady state free precession (bSSFP) imaging sequence but they were not visible with Gd-DTPA.

Brain metastases due to melanoma have not been studied longitudinally in vivo. Leenders et al. used Gd-DTPA enhanced MRI to demonstrate that vascular endothelial growth factor-A (VEGF-A) expressing melanoma brain metastases were visible after Gd-DTPA injection due to leaky peritumoral vasculature but brain metastases that did not express VEGF-A went undetected (19). This study looked at the brain metastases at a single time point. Longitudinal imaging allows the integrity of the BBB to be monitored over time to observe how it changes with tumour development and in response to therapy.

In the following study we used longitudinal MRI to characterize a clinically relevant model of melanoma brain metastases and we applied the imaging approach developed by Percy et al. (17) to assess BTB integrity and total metastatic burden at four different time points during metastasis development. Our goal was to determine what percentage of melanoma brain metastases were permeable to Gd-DTPA, and therefore considered to have a leaky BTB, and to learn more about when and why certain brain metastases are more permeable than others.
2.2 Materials and Methods

2.2.1 Experimental groups

Two experiments were performed which used the methods described below. The goal of experiment 1 was to characterize the MRI appearance, number and size of brain metastases that developed approximately one month following the intracardiac injection of 50,000 A2058 human melanoma cells into nude mice. Nine mice were imaged once, at the endpoint of the experiment. Experiment 2 was designed to monitor the development of these brain metastases over time in two groups of mice. This included MRI methods that measured the total brain metastatic burden and evaluated the BTB integrity at multiple time points. Two groups of 4 mice were scanned at multiple time points post cell injection.

2.2.2 Cell culture and cell viability assays

The A2058 human metastatic melanoma cell line was obtained from American Type Culture Collection (ATCC)® (cell line provider Cedarlane Labs, Canada). Cells were cultured at 37°C, 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) (Gibco® Invitrogen). Cells were passaged every 2-3 days.

A2058 cells were incubated overnight with micron sized paramagnetic iron oxide (MPIO) nanoparticles that were conjugated to either Flash Red (660nm excitation and 690nm emission spectra) or Dragon Green (480nm excitation and 520nm emission spectra) (Bangs Laboratories, USA). The green fluorescent MPIO was used for experiments where we also perfused mice with a red fluorescent dextran probe (see below). A trypan blue exclusion assay was performed prior to and after intracardiac cell injections for both MPIO-labeled and unlabeled cells to measure cell viability. Cytospins were performed for unlabeled and MPIO-labeled cells and Perls Prussian Blue (PPB) staining for iron was performed to assess MPIO uptake.
A cell proliferation assay was performed to determine the impact of MPIO labeling on cellular function. The A2058 melanoma cells were plated at a density of $2 \times 10^5$ cells/ml in both T75 (cell culture flask with 75cm$^2$ growth surface area) and T25 (cell culture flask with 25cm$^2$ growth surface area) flasks. Two different flasks were used to see which housed the best environment for the experiment and the optimum growth of cells. Four cell culture flasks containing unlabeled and labeled (two T75 and two T25) A2058 human melanoma cells were counted manually using a hemocytometer. Unlabeled and labeled A2058 cells were counted on days 0, 2, 5 and 7 to generate a growth curve showing cell proliferation. Growth rates were determined from the linear regression for the exponential growth curve equation generated in Microsoft Excel software. The formula for the exponential growth curve was as follows: $y = ae^{bt}$ where $y$ = cell number after time $t$, $a$ = the initial cell number, $b$ = the growth constant (in this case units are day$^{-1}$), $t$ = time (in this case days).

2.2.3 Animal Model

Athymic immunodeficient female nude mice (strain #088 Foxn1 Nu/Nu) age 6-8 weeks old were used (Charles River laboratories, Saint Constant, Quebec) in accordance with an approved animal use protocol (Appendix A: Animal Use Protocol). Mice were housed in a barrier facility at the Animal Care and Veterinary Services (ACVS) at Western University. For cell injections, mice were anesthetized with a mixture of 100% oxygen (flow rate 2 litres per minute) and isofluorane (3% for induction, 1-1.5% for maintenance). A U-100 insulin syringe with a 28G ½ needle (Becton Dickinson) was used to inject 50,000 MPIO-labeled A2058 cells in 100µl Hank’s Balanced Salt Solution (HBSS) into the left ventricle of the beating mouse heart. Mice were weighed and monitored weekly for signs of health or sickness (disease onset was prevalent at week 3 and more pronounced at week 4 when mice became moribund and cachexic due to the aggressiveness of malignant melanoma).

2.2.4 Imaging

All mice were imaged using a 3 Tesla (T) General Electric (GE) MR750 MRI clinical scanner equipped with a custom-built gradient coil insert (maximum gradient coil
strength = 500 mT/m and maximum slew rate = 3,000 T/m/sec) and a 1.5-cm diameter solenoidal radiofrequency (RF) coil for the mouse head. Before scanning, mice were anesthetized with 3% isofluorane for induction and lowered to 1-2% for maintenance. During scanning, all mice were kept warm through exploitation of heat convection (heated saline bags were attached to the insert tray of the gradient coil).

To evaluate the success of the intracardiac cell injection, mice were imaged using the balanced steady-state free precession (bSSFP) imaging sequence. The following parameters were used: Field of View (FOV) = 2.0 cm, repetition time (TR) = 16 ms, echo time (TE) = 8 ms, flip angle (FA) = 35°, image matrix = 100 x 100, number of excitations (NEX) = 1, receiver bandwidth (rBW) = ±41.67 kHz, phase cycles (PC) = 4. The scan time was 13:41 min and the spatial resolution was 200 x 200 x 200 µm.

For experiment 1, higher resolution bSSFP images were acquired on day 25 (n=5) or day 26 (n=4) post cell injection. The following imaging parameters were used: FOV = 1.5 cm, TR/TE=8/4 ms, FA = 35°, image matrix = 150 x 150, slice thickness = 200 µm, NEX = 2, rBW = ±41.67 kHz, PC = 8. The scan time was 30:45 min and the spatial resolution was 100 x 100 x 200 µm. These images were used to count the number of metastases and to measure the volume of each metastasis.

For experiment 2, two imaging sequences were used on consecutive days at four different time points: days 21/22 post cell injection, days 24/25 post cell injection, days 27/28 post cell injection, and days 30/31 post cell injection. On the first day of each time point T1 weighted spin echo (T1wSE) images were acquired after the intraperitoneal (IP) injection of Gadopentatic acid (Gd-DTPA; 200µl undiluted, Magnevist™, Schering, USA) to assess the integrity of the BBB. Images were acquired 60 minutes post Gd-DTPA injection. The following parameters were used: frequency FOV = 2 cm, phase FOV = 1 cm, TR = 600 ms, TE = 20 ms, image matrix = 128 x 128, NEX = 12, rBW = ±15.63 kHz. The scan time was 15:45 min and the spatial resolution was 156 x 156 x 500 µm. On the second day, bSSFP
images were acquired for the same mice to assess total metastatic tumour burden. The imaging parameters were the same as for bSSFP images acquired in experiment 1. During scanning of the first group of 4 mice, some mice had to be sacrificed and these were replaced with others, so that 4 mice were imaged at each time point although these were not always the same 4 mice. For the second group of mice all four animals were imaged 8 times each, at all of the pre-determined time points.

2.2.5 Image analysis

OsiriX™ image software (open source) was used for image visualization and analysis. The bSSFP images were used to count the number of metastases and to measure the volumes of individual metastases within the whole brain; this represented the total number of metastases (see Appendix C for total metastatic burdens of mice). The volumes of metastases were only measured for the first group of mice in experiment 2. In total the volumes of 120 individual metastases were calculated. To measure the volume, a manual trace method was used in which tumour borders were manually outlined in each image slice and a final algorithmic computation was performed to obtain a three-dimensional (3D) tumour volumetric measurement (mm$^3$). The T1wSE images were used to determine which metastases enhanced after Gd-DTPA. T1wSE images were viewed along with bSSFP images at all four time points. Enhancing metastases were defined as those with an increase in signal intensity hyperintensity compared to the surrounding mouse brain tissue, in regions where bSSFP images revealed a metastasis. Metastases classified as nonenhancing were not visible in T1wSE images but were present in bSSFP images.

Due to differences in coverage of the mouse brain for both sequences not all enhancing metastases were evaluated for the T1wSE scan portion. Enhancing metastases were evaluated from frontal to hindbrain, which began at slice 1 and ended at slice 11 for T1wSE images. bSSFP images that had a total of 184 image slices were compared simultaneously with T1wSE post Gd-DTPA images. The image slices for bSSFP were matched according to the start and end image slices for the T1wSE post Gd-DTPA images. Some metastases were
visible in bSSFP images but were not evaluated as enhancing or nonenhancing because there was no corresponding T1wSE post Gd-DTPA image.

Parenchymal metastases were defined as those located within the brain tissue. Metastases were classified as meningeal if at least one side of the metastasis appeared to be located along the surface of the brain. To help verify that metastases were meningeal OsiriX™ image software was used. In OsiriX™, the orthogonal reslice tool was utilized for reslicing the acquired axial MRI images in the X (sagittal) and Y (coronal) planes. Scrolling through the image slices in X and Y planes were performed to confirm the status of the metastasis as a meningeal metastasis. Meningeal metastases were further classified as originating from either the pia (leptomeninges) or dura mater according to the MRI appearance. The observation of cerebrospinal fluid (CSF) between the brain tissue and the tumour suggested that the metastasis had originated from the dura mater.

2.2.6 Histology and Microscopy

Mice were sacrificed after scanning and brains were prepared for microscopy. Brains were either embedded in paraffin or Optimal Cutting Temperature (O.C.T) medium. For paraffin embedding, mice were perfused with 4% paraformaldehyde (PFA) and then brains were removed and cut into sections with image guidance then placed in tissue embedding cassettes. The cassettes were placed in 70% ethanol before paraffin embedding. Using a microtome, brains were sectioned into 10-µm tissue sections and two sections per slide were placed on positive glass slides. Slides were stained with Hematoxylin and Eosin (H&E) using an automated stainer (Leica Microsystems).

For frozen tissue sections, mouse brains were removed after perfusion and placed in increasing concentrations of sucrose 10%, 20%, 30% (overnight) to cryoprotect tissues before embedding. Mouse brains were embedded (positioned dorsal to ventral) in O.C.T medium with the ventral side facing the bottom surface of the cryomold. Using a cryostat
Leica Microsystems) frozen sections were cut at a thickness of 20-µm with four sections placed on positive glass slides. Slides were placed in a -20°C freezer for long-term storage.

For some mice an *ex vivo* permeability assay was performed. Four mice were anaesthetized with 3% isofluorane and perfused via tail vein injection with 200µl of 1.5 mg Dextran Texas Red (this molecular dye can only cross a non-intact BBB). The Dextran Texas Red was allowed to circulate for 20min prior to mouse sacrifice. After sacrificing, mice were perfused with 4% PFA and brains were removed and frozen tissue sections were prepared as described above. Prior to tissue sectioning, mouse brains were temporarily stored at -80°C. After sectioning, the remaining frozen tissue was stored at -20°C.

Images of H&E stained and fluorescence frozen sections were acquired with a Zeiss Axio Imager A1 microscope (Zeiss, Germany) using a Retiga Exi (QImaging, Canada) digital camera. Image Pro Plus software Version 7 was used for histological image editing. For fluorescence image analysis, slides with frozen mouse brain sections were analyzed first for dextran leakage. After acquiring Dextran Texas Red (595nm excitation and 615nm emission spectra) fluorescence images, frozen tissue sections were H&E stained using an automated slide stainer (Leica Microsystems). Both H&E images (paraffin and frozen sections) as well as fluorescence images were matched as close as possible with MRI images. An exact match is difficult to achieve due to differences in slice thicknesses for MRI and histology.

2.2.7 Statistical Analyses

Statistics were performed using GraphPad Prism® software Version 5.0. The numbers of metastases in the characterized model were represented as mean ± Standard Deviation (SD). Comparison of meningeal vs. parenchymal brain metastases was performed using unpaired student t-tests; data was represented as mean ± Standard Deviation (SD). Comparison of groups (enhancing vs. nonenhancing brain metastases) at each time point during the longitudinal studies was performed using the Kruskal-Wallis test followed by the posthoc test: Dunn’s Multiple Comparison Test. The level of statistical significance was p<0.05.
2.3 Results

2.3.1 Cell Labeling

To visualize cells with MRI they were labeled with iron oxide contrast agent. Figure 2.3.1 shows images of PPB stained cells for unlabeled (A) and MPIO-labeled (iron-labeled) (B) A2058 cultured cells. After PPB staining, nearly all labeled A2058 cells stained positively for iron, as shown by blue punctate iron deposits. Without MPIO labeling, A2058 cells did not stain blue after PPB. The cell viability prior to injection was 93.8% for unlabeled A2058 cells and 95.8% for MPIO-labeled A2058 cells. Cell viability after cell injection was 97.8% for unlabeled A2058 cells and 89.5% for MPIO-labeled cells. To examine the effects of iron labeling on the proliferative potential of A2058 cultured cells, a proliferation assay was performed. Cell numbers for unlabeled and MPIO-labeled A2058 melanoma cultured cells were generated based on manual cell counts at days 0, 2, 5 and 7.

![Fig 2.3.1 Perls Prussian blue staining of iron-labeled and unlabeled human melanoma A2058 cultured cells. (A) Unlabeled (no iron present) and (B) MPIO-labeled (which are iron-labeled) A2058 human melanoma cells. Scale bars = 100µm](image)

Figure 2.3.2 shows the growth curves for unlabeled and MPIO-labeled melanoma cells. Overlap of the exponential curves is observed indicating that growth rates were similar for unlabeled and labeled cells (grown in T75 flasks and T25 flasks) at all four time points. The grow rate constants were as follows 0.3581 day\(^{-1}\) (unlabeled cells T25 flask), 0.4008 day\(^{-1}\)
(labeled cells T25 flask), 0.3901 day\(^{-1}\) (unlabeled cells T75 flask) and 0.4043 day\(^{-1}\) (labeled cells T75 flask). Thus growth rates were similar for unlabeled and MPIO-labeled melanoma cells irrespective of the cell culture flask, growth area and iron labeling. The cell viability was also measured at each time point, by trypan blue exclusion, and varied from 88 to 100%.

Fig 2.3.2. Cell proliferation assay. Growth curves for unlabeled and Bangs (MPIO) labeled A2058 melanoma cells. All four plots show similar growth rates indicating that iron labeling did not affect this biological function of these cells.

2.3.2 Imaging

To confirm that the intracardiac cell injections were successful, short bSSFP scans (short scan time) were acquired and the brain images evaluated for the presence of signal voids, which represent iron-labeled cells. Figure 2.3.3 shows a representative bSSFP image slice obtained on day 1 post cell injection where numerous discrete regions of signal void (white arrows) throughout the brain indicate a successful injection. The success rate for this experiment (referring to experiment 1) was 90% (9/10 mice).
Figure 2.3.4 shows the appearance of melanoma metastases in the mouse brain in bSSFP images. Compared to the surrounding brain tissue, melanoma brain metastases in this mouse brain appeared with increased signal intensity (hyperintense). Both parenchymal and meningeal metastases were detected throughout the brain. Parenchymal metastases were characterized as those located within, and surrounded by, the brain tissue and having no contact with the surfaces of the brain at first detection. Examples of parenchymal metastases for the mouse brain shown in figure 2.3.4 can be seen in A, D&G (white arrows). Meningeal metastases were defined as those with at least one side adjacent to the surface of the brain when first detected. There were two different types of meningeal metastases observed. The first type of meningeal metastasis appears to originate from the outer meninges, the dura mater (refer to figure 2.3.5). In MRI images these metastases are located between the skull and CSF. The second type of meningeal metastasis appears to originate from the inner meningeal layers (leptomeninges). Examples of meningeal metastases for the mouse brain shown in figure 2.3.4 can be seen in C, E&F (yellow arrows). Figure 2.3.4 also shows examples of parenchymal metastases for the mouse brain that can be seen in A, B, D & G-I.
Enlarged ventricles (figure 2.3.4 D, E, and F) were also observed. There was a wide range of volumes for both parenchymal and meningeal metastases in this mouse brain. The mean volume of all metastases was 0.532 mm$^3$. The volumes did not appear to depend on brain location. The largest metastasis measured was 2.47 mm$^3$ and the smallest metastasis measured was 0.0438 mm$^3$; both were located in the hindbrain.

**Figure 2.3.4 bSSFP images showing the appearance and spatial distribution of melanoma metastases in a representative mouse brain.** Representative bSSFP axial image slices of brain metastases (both meningeal; yellow arrows and parenchymal; white arrows) from the same mouse. All metastases (A-I) appeared hyperintense compared to the surrounding brain tissue. In (D, E &F), the brain ventricles appeared hyperintense and were enlarged.

Another example of a meningeal metastasis (yellow arrow) that appeared to originate from the dura mater is shown in figure 2.3.5 below. This metastasis appeared to be compressing and displacing the normal brain tissue.
Figure 2.3.5 bSSFP image of a melanoma meningeal metastasis in a mouse brain. bSSFP image of a melanoma metastasis (yellow arrow) that appeared to be growing from the dura mater (most outer layer) of the brain meninges.

MRI detectable metastases were counted for 9 mice, n=5 mice from images acquired at day 25 and n=4 mice from images acquired at day 26. Overall, the number of metastases that developed at these endpoints ranged from 5 to 19 per mouse brain. The average number of metastases per mouse brain was 11 (refer to figure 2.3.6).
Fig 2.3.6 Numbers of melanoma brain metastasis after 50,000 cells injected. A cell injection of 50,000 A2058 melanoma cells produced between 5 and 19 brain metastases. 5/9 mice developed more than 10 brain metastases and 4/9 mice developed fewer than 10 metastases at days 25 and 26. Data represented as mean ± SD.

The mean number of meningeal and parenchymal brain metastases that developed is shown in figure 2.3.7. Significantly more parenchymal metastases were observed than meningeal metastases (approximately 80% were parenchymal). See Appendix D for total, mean metastasis numbers and volumes for meningeal and parenchymal brain metastases.
Fig 2.3.7 Mean number of meningeal and parenchymal melanoma brain metastases. Using an unpaired t-test, a statistically significant difference (p=0.013) was evident amongst the mean parenchymal (9.00 ±1.74) and mean meningeal (2.44±0.626) metastases. Data are mean ± SD

H&E staining of the brain sections showed that meningeal and parenchymal metastases had similar morphologies; they were hypercellular and had little associated edema. In Figure 2.3.8 the MRI is shown (A) alongside a low power H&E staining of the corresponding whole brain section (B). A meningeal metastasis (box in B) is shown at 5x magnification in (C). This metastasis appeared as a tight cluster of cells with little associated edema. At 100x magnification (box in C) groups of melanoma cells are seen (D).
Figure 2.3.8 MRI and H&E histology for A2058 melanoma meningeal brain metastasis. (A) bSSFP MRI showing a meningeal metastasis (white arrow) and (B) whole brain H&E that correlates to MRI, scale bar = 1mm. Meningeal metastasis (C) at 5x magnification, scale bar = 1mm. (D), 100x magnification (inset C) showing groups of melanoma cells, scale bar = 50µm.

Figure 2.3.9 shows an MRI image of a parenchymal metastasis (A) and the corresponding whole brain section H&E staining is shown in (B). The H&E section at 5x (C) shows a hypercellular mass of cells that was located next to the left lateral ventricle (white arrow). At 100x magnification (D), the black arrow points to what appeared to be a small area of edema in between the melanoma cells.
Figure 2.3.9 MRI and H&E histology for A2058 melanoma parenchymal brain metastases. The bSSFP MRI (A) image shows parenchymal brain metastases as hyperintense areas (white arrows). (B) Shows the corresponding whole brain H&E section scale bar = 1mm. (C) One of the metastases (box in B) is shown at higher magnification scale bar = 1mm. The white arrow in (C) points to the choroid plexus located in the left lateral ventricle. (D) The black arrow points to what appears to be edema surrounded by melanoma cells (box in C), scale bar = 50µm.

2.3.3 Longitudinal MRI

The model of melanoma brain metastasis characterized above was implemented in a longitudinal MRI experiment to monitor the development of brain metastases and to evaluate the integrity of the BTB over time. Mice were imaged in two groups so that the amount of time to image all mice on each day was more reasonable (the time required to scan 5 mice with bSSFP was approximately 5 hours and the time required to scan 5 mice with T1wSE was approximately 4 hours). The total tumour burden was determined by counting all metastases in bSSFP images (see Appendix C for total metastatic burden of mice at each time point) at the endpoint of the longitudinal experiment (day 31) (which was later than for the
characterization experiment; days 25/26), we observed a range of 4 to 20 brain metastases per mouse. Mice with more than 20 brain metastases (n=3) did not survive until this endpoint. Approximately 85% of the metastases were determined to be parenchymal.

Figure 2.3.10 shows T1wSE post Gd-DTPA and bSSFP images for a representative mouse. (A&B) are T1wSE post Gd-DTPA images and C&D are bSSFP images. The images shown in (A&C) were obtained on days 24 and 25 and the images shown in (B&D) were acquired on days 27 and 28. A single metastasis was visible in the bSSFP image on day 25 but did not appear in the T1wSE post Gd-DTPA image acquired the day before. At the next imaging session (B&D), the same metastasis appeared in both the bSSFP image and T1wSE post Gd-DTPA image. This example showed that there are metastases that do not enhance after Gd-DTPA, and therefore are not associated with a disrupted BTB, but are detectable in bSSFP images. It also showed that the integrity of the BTB associated with melanoma brain metastases can change over time.
Figure 2.3.10 bSSFP and T1wSE post Gd-DTPA images of brain metastases. T1wSE+Gd-DTPA and bSSFP at early (days 24/25) and later time points (days 27/28) show the sensitivity of bSSFP to brain metastases (nonenhancing) that were undetectable using T1wSE+Gd-DTPA at the early time point. In (C) a single brain metastasis at day 25 was visible in the bSSFP image but not in the T1wSE post Gd-DTPA acquired the day before. At day 27 (B) this metastasis was enhancing and in the bSSFP image (D) appeared to have increased in size. Some metastases were detected in bSSFP but not in T1wSE post Gd-DTPA images.

Another interesting observation was that within mice there was substantial heterogeneity in the number of enhancing metastases. Figure 2.3.11 illustrates this finding. A-D are T1wSE post Gd-DTPA images and E-H are bSSFP images. In (A) at day 21, only one enhancing metastasis was visible. This same metastasis was also visible in bSSFP images acquired on day 22 (E). At the following imaging session on day 24 (B) the same enhancing metastasis was still visible in the post Gd-DTPA image and had increased in size, however, two metastases were now visible at day 25 in the bSSFP image (F). At the next imaging session C&G, the same metastases were observed. The larger metastasis at the base of the brain was a meningeal metastasis and the smaller metastasis towards the top of the brain was parenchymal. At the final imaging sessions day 30 (D) and day 31 (H), two enhancing metastases were detected in the T1wSE post Gd-DTPA image and three metastases were
visible in the bSSFP image (H). This example showed that multiple metastases could be found within the same mouse brain with varying Gd-DTPA enhancement profiles.

![Image of MRI scans showing brain metastases]

**Figure 2.3.11 Heterogeneity in enhancement for A2058 melanoma brain metastases.** MRI images A-D are T1wSE+Gd-DTPA images and E-H are the corresponding bSSFP. One metastasis was seen as enhancing from days 21 to 27 (A-C). At day 30, a second metastasis was visible as enhancing (near the top of the brain). In the bSSFP images (E-H) the number of metastases increased over time. At day 31 (H) three metastases were detected but only two metastases were visible as enhancing at day 30 (G).

In Table 2.3.1 the number of metastases that enhanced at each time point are presented for each of the mice. This is reported as X of Y where X is the number of enhancing metastases in T1wSE post Gd-DTPA images and Y is the number of metastases identified by bSSFP. Note that the number of metastases identified by bSSFP, reported in this table, is not the total metastatic burden (see Appendix C for total metastatic burden in mice at each time point). At the first imaging time point only 2 of 9 mice (mice 6 and 7) had metastases that enhanced in T1wSE post Gd-DTPA images; each mouse had just one enhancing brain metastasis. At the second imaging session enhancing metastases were detected in 7 of the 9 mice. At the last imaging session all of the surviving mice had more than one enhancing metastasis. One mouse had no enhancing metastases throughout the entire experiment (mouse 3). This mouse was sacrificed early due to signs of poor health.
With the exception of one animal (mouse 5) the percentage of metastases that enhanced increased with time. Mouse 5 was a unique case, the number of enhancing metastases changed from 6 out of 14 to 3 out of 14, between the third and fourth imaging sessions, because a prominent area of signal void developed within 3 of the metastases, most likely due to hemorrhage, obscuring the visualization of the metastases. Enhancing metastases never changed to nonenhancing metastases in our study. Two mice died during the scanning sessions on day 25; both had relatively high metastatic burden. The images also showed that they had enlarged ventricles (hydrocephalus). Both of these mice had very large meningeal metastases at the base of the brain. Furthermore, the volume of the metastases measured in these mice at day 25 was significantly larger than those of the other mice at this time point. Mouse 2 and mouse 4 had the fewest metastases at endpoint and also the highest percentage of enhancing metastases.

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Numbers of Metastases that Enhanced at Each Timepoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan Days</td>
<td>Day 21/22</td>
</tr>
<tr>
<td>Group 1</td>
<td>Mice</td>
</tr>
<tr>
<td>1</td>
<td>0 of 3</td>
</tr>
<tr>
<td>2</td>
<td>0 of 1</td>
</tr>
<tr>
<td>3</td>
<td>0 of 3</td>
</tr>
<tr>
<td>4</td>
<td>0 of 1</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1 of 2</td>
</tr>
<tr>
<td>7</td>
<td>1 of 10</td>
</tr>
<tr>
<td>8</td>
<td>0 of 1</td>
</tr>
<tr>
<td>9</td>
<td>0 of 2</td>
</tr>
</tbody>
</table>

Table 2.3.1 Number of enhancing melanoma brain metastases over time.

bSSFP images also showed regions of low signal that developed within metastases over time. Figure 2.3.12 shows bSSFP images obtained on days 22, 25, 28 and 31 post cell injection. Two metastases were present; a parenchymal metastasis was visible at day 22 as a region of signal hyperintensity (yellow arrow) and a different parenchymal metastasis (blue arrow) remained isointense throughout the study (day 22 to day 31), this metastasis was not visible
in bSSFP images but was identified in histological analysis (refer to figure 2.3.12, E). At day 25, the metastasis (yellow arrow) appeared to increase in size and a small dark void appeared within it. At day 28 the metastasis (yellow arrow) and the region of signal hypointensity were both larger. By day 31 the metastasis (yellow arrow) itself was no longer visible in this image slice. Instead, what is referred to as a blooming artifact, appeared in its place, the result of a large accumulation of superparamagnetic species which the H&E staining for this metastasis indicated was due to hemorrhage. The bottom panel shows the corresponding whole brain H&E staining (E) to the bSSFP image in (D). (F) Shows the 40x magnification of the hemorrhagic metastasis from the box in E. The 100x magnification in (G) is a region in (F) that shows the presence of blood encircling and nested within the melanoma cells (white arrows).

An important observation was made during the comparison of MRI and histology. In several mice, metastases that were detected in H&E histology were not obvious in either bSSFP or T1wSE images. When the images were evaluated retrospectively we found that some of these metastases appeared with the same signal intensity as the surrounding brain tissue in bSSFP images (isointense). An example of an isointense brain metastasis can be seen in Figure 2.3.12 (blue arrows).
Figure 2.3.12 bSSFP images show development of a hemorrhagic metastasis and nonhemorrhagic metastases. Top panel: bSSFP images from the same mouse. (A-D) shows development of a parenchymal brain metastasis (yellow arrows) in the frontal brain region at four time points. The metastasis increased in size over time and started to show signs of signal loss (dark circle in center) at day 25. At day 31 the metastasis was no longer visible due to the blooming artifact. The numbers in white (lower right of each panel) represent the total metastatic burden at each time point indicated. The blue arrows point to a metastasis that appears isointense in bSSFP images. Bottom panel: (E) whole brain H&E corresponding to (D top panel), blue arrow points to the metastasis that is visible in the H&E image but is isointense (blue arrows) in bSSFP images (A-D). Two nonhemorrhagic metastases are also visible near the top left hemisphere of the brain, scale bar = 500µm, (F) 10x magnification of hemorrhage from box in A scale bar = 500µm. (G) Hemorrhage was observed (white arrows) in the metastasis from (F), scale bar = 100µm.
The volumes of metastases from experiment 2, group 1 (see table 2.3.1), were measured at each time point from bSSFP images (for reliability of volume measurements refer to Appendix B for inter- and intra-observation (35,36) volume measurements of melanoma brain metastases). This is presented in Figure 2.3.13. There was a large range of volumes for metastases at each time point. The volumes increased over time for both enhancing and nonenhancing metastases. In these 4 mice (from experiment 2, group 1) there were no enhancing metastases detected at day 21. The smallest MRI detectable metastasis was 0.0143 mm$^3$ and was detected at day 25. The largest metastasis was 42.9 mm$^3$ and was detected at day 31. There was no statistically significant difference in the volumes of enhancing and nonenhancing metastases at any time point.

![Figure 2.3.13 Volumes of enhancing and nonenhancing of A2058 melanoma brain metastases at each time point. Although a trend for increasing volume was seen over time, there was no statistical difference (p<0.05) in volumes of enhancing and nonenhancing metastases. Bars represent medians.](image-url)
Figure 2.3.14 shows bSSFP images for both small and large enhancing and nonenhancing metastases.

**Figure 2.3.14** Representative bSSFP images comparing volumes for nonenhancing and enhancing melanoma brain metastases from the same mouse. A&B show enhancing metastases (with their accompanying volumes) in the midbrain-hindbrain (A), and frontal regions (B). C&D show nonenhancing metastases in olfactory-frontal (C), and frontal regions (D).

Figure 2.3.15 illustrates some of the different patterns of enhancement over time for individual metastases. For example, some metastases were enhancing when they were first detected and remained enhancing throughout the study (light blue line), some metastases never enhanced (pink line), many metastases first appeared as nonenhancing but changed to enhancing at later time points (light red, dark blue and dark purple lines). We also observed new metastases that appeared at the last imaging time point (red, green and light purple dots); these late appearing metastases were all nonenhancing. This figure also shows that the volume of a metastasis did not predict whether or not it would be an enhancing metastasis. For instance at the late time point (day 31) large enhancing and nonenhancing metastases were observed as well as small enhancing and nonenhancing metastases. Metastases that were first detected at the last imaging session were always nonenhancing.
Figure 2.3.15 Differences in patterns of enhancement over time for A2058 melanoma brain metastases. Each line represents a different metastasis. Individual metastases showed varying enhancement patterns as shown by the colored lines; light blue line = metastasis first detected as enhancing and remained enhancing, pink line = metastasis that never enhanced, light red, dark blue and dark purple line = metastases first appeared as nonenhancing but changed to enhancing at later time points. Red, green and light purple dots = late appearing metastases were nonenhancing. Hollow circles represent enhancing metastases and solid circles represent nonenhancing metastases.

We compared our in vivo permeability data obtained with T1wSE post Gd-DTPA images with the ex vivo permeability using Dextran Texas Red; this dye can only leak through a non-intact BBB. Figure 2.3.16 shows T1wSE post Gd-DTPA (A) and bSSFP (B) images of an enhancing metastasis (white arrows) and the corresponding dextran fluorescence and H&E images (C&D) for this single metastasis. The fluorescence image (C) shows the presence of red fluorescence associated with the tumour, which suggests that this metastasis was permeable to the dextran. The corresponding H&E shows that the enhancing metastasis was hypercellular.
Figure 2.3.16 Representative T1wSE post Gd-DTPA and bSSFP images of an enhancing metastasis with corresponding dextran fluorescence image and H&E histology. (A) T1wSE post Gd-DTPA image. (B) bSSFP image. (C) 10x magnification dextran fluorescence image of an enhancing metastasis (A, white arrow) showing dextran leakage within the metastasis, scale bar = 971µm. (D) H&E image showing 10x magnification of the enhancing metastasis (white arrow, A), scale bar = 971µm.

Figure 2.3.17 shows T1wSE post Gd-DTPA (A) and bSSFP (B) images of a nonenhancing metastasis (white arrow). There appeared to be minute dextran fluorescence (C) in the tumour interstitial space suggesting that BTB regions within this metastasis were not completely intact.
Figure 2.3.17 Representative T1wSE post Gd-DPTA and bSSFP images of a nonenhancing metastasis with corresponding Dextran and H&E histology. (A) T1wSE post Gd-DTPA image. (B) bSFFP image. (C) 10x magnification dextran fluorescence image of a nonenhancing metastasis from (B, white arrow) showing little to no dextran leakage within the metastasis, scale bar = 971µm. (D) H&E image showing 10x magnification of metastasis in (B), scale bar = 971µm.

Figures 2.3.18 shows enhancing and nonenhancing metastases that were situated in different brain locations. Figure 2.3.18 shows T1wSE post Gd-DTPA images (A&D) and bSSFP images (B&E) at day 31. The insets (B&E) show enhancing and nonenhancing metastases respectively located in different brain locations; frontal-midbrain (B) and midbrain brain region (E). (C&F) are the corresponding H&E 10x magnifications from the boxes in (B&E). Hypercellularity was observed for both enhancing and nonenhancing metastases.
Figure 2.3.18 Representative T1wSE post Gd-DTPA and bSSFP images of an enhancing and nonenhancing metastasis with corresponding H&E histology. A&D T1wSE post Gd-DTPA images and B&E bSSFP images. (A) Enhancing metastasis (blue arrow), (B) corresponding bSSFP metastasis to (A, blue arrow), (D) no visible enhancing metastasis in T1wSE with Gd-DTPA, (E) nonenhancing metastasis visible with bSSFP. (C) H&E image of an enhancing metastasis in the frontal-midbrain region showing hypercellularity, scale bar = 971µm. (F) H&E image of a nonenhancing metastasis in the midbrain region, showing hypercellularity, scale bar = 971µm.
2.4 Discussion

In the present study we have established a clinically relevant model of melanoma brain metastases and characterized the appearance and size of metastases using MRI. To the best of our knowledge this is the first study to use MRI to monitor the development of melanoma brain metastases longitudinally. This is also the first report of the use of bSSFP to image melanoma brain metastases. This model was achieved using an injection of 50,000 A2058 human melanoma cells into the left ventricle of the heart in nude mice and resulted on average, 13 brain metastases per mouse. Metastases were detected throughout the mouse brain; the majority was parenchymal, but meningeal metastases were also common.

There are relatively few animal models of brain metastases. The more conventional experimental metastasis models use tail vein injection for hematogenous delivery and most cells are trapped in the lung; the first organ encountered with an extensive capillary bed. Brain metastases rarely develop, in part because mice with lung metastases do not survive long enough. This issue is circumvented with an intracardiac (IC) injection. The initial distribution of cells after the IC injection depends on cardiac output, therefore a portion of the cells colonize the brain as it is the first capillary bed to be reached. This model captures several mechanisms fundamental to metastatic growth: survival in the circulation, arrest in a secondary organ, extravasation into the target organ tissue and colonization in the distant metastatic site. We have previously used this approach to study brain metastases due to breast cancer (37-40). The Steeg lab, with whom we collaborate, has used this model extensively to study the molecular mechanisms and treatments for brain metastasis (41-44). The IC cell injection method has been used to induce melanoma brain metastases in a small number of studies, mostly using the B16 mouse melanoma cell line (45,46). Other models of melanoma brain metastasis have used an intracarotid (ICA) cell injection or direct intracranial implantation of cells, both of which require surgical manipulation (18,47-54).

MRI was used to determine the types, numbers and volumes of brain metastases due to melanoma. In bSSFP images most melanoma brain metastases appeared slightly or
moderately hyperintense compared to the surrounding brain tissue. Occasionally, metastases appeared hypointense, more often at late time points. Our histology also revealed that not all metastases were detected by bSSFP MRI, and looking back retrospectively we found that some metastases were isointense with the surrounding brain tissue and therefore inconspicuous. This presents as a limitation in our study since metastatic burden is higher than what is previously reported in our study at various imaging time points. The variable MRI appearance of melanoma brain metastases is consistent with what is reported clinically and is based largely on the degree of melanin and the presence or absence of hemorrhage (55-58).

In patients, classic melanotic melanoma appears hyperintense on T1wSE images and hypointense on T2wSE (57). This is because melanin is a paramagnetic species, which shortens both T1 and T2 relaxation times (in SE images shorter T1 means brighter, shorter T2 means darker). Since the amount of melanin varies between melanoma brain metastases the amount of contrast can vary. Amelanotic melanoma metastases appear hypo- or iso-intense on T1w images and hyper- or iso-intense on T2w images (57).

Hemorrhage has strong effects on the MR signal and contrast due to hemorrhage is even more complicated because as the hemorrhage evolves the oxidation state of the hemoglobin molecule changes and the red blood cell (RBC) membranes lyse (59-61). It is the iron moiety of the hemoglobin that influences changes in the MR signal. In the early subacute stage (few days), deoxyhemoglobin (deoxy-Hb) in the intact RBC oxidizes to methemoglobin (Met-Hb). Iron is in the ferric oxidation state (Fe$^{3+}$); it has five unpaired electrons and is paramagnetic. In addition the configuration of Met-Hb allows water molecules to come into close contact with the unpaired electrons. At this stage Met-Hb is intracellular. Water molecules move freely across the cell membrane and can thus approach close enough for T1 shortening effects to occur. In addition the inhomogeneous distribution of intracellular metHb results in local magnetic field inhomogeneities and so T2 relaxation shortening. In the late subacute stages (4-7 days to 1 month), RBC cell lysis occurs and Met-Hb is now extracellular; Met-Hb is surrounded by water protons, which leads to profound T1 shortening. However, because
Met-Hb in solution is homogeneously distributed T2 shortening is less significant. At the chronic stage of hemorrhage (weeks to years) hemosiderin is present in macrophages. Hemosiderin is insoluble and has a large number of unpaired electrons and is superparamagnetic. Because it is insoluble in solution water protons cannot approach close enough for T1 shortening effects to occur. Conversely, the inhomogeneous distribution of this powerful superparamagnetic leads to profound T2 shortening. Two or more of the hemoglobin compounds are often present concurrently in a hemorrhage making MRI contrast complex.

Contrast in bSSFP images is related to T2/T1. Our observation of slightly or moderately hyperintense brain metastases could be due to melanin content and/or hemorrhage. Depending on the melanin content, the degree of T1 shortening (hyperintensity) will be variable. (See Appendix G for melanoma brain metastases that contain melanin pigmentation). The observation of isointense metastases might be due to simultaneous T1 and T2 shortening. Alternatively this could be due to the absence of melanin (amelanotic) and blood degradation products in brain metastases. The hypointense metastases we observed with bSSFP are most likely due to blood degradation products such as hemosiderin. Due to observation of hemorrhage from histological analysis it would suggest that hemorrhage is most likely the main contributor to a decrease in signal.

Through the use of two MRI sequences, bSSFP and Gd-DTPA-enhanced T1wSE, we were able to evaluate the metastatic tumour burden and the integrity of the BTB over time as metastases developed in the brain. There were two major findings from this work. First, there is considerable heterogeneity in the permeability of melanoma brain metastases in this model. Second, the bSSFP sequence enabled the detection of brain metastases that were not detected (i.e. did not enhance) in post Gd-DPTA T1wSE images.

The presence of substantial heterogeneity of the BTB in our study suggests that differences in permeability may have an effect on how well chemotherapeutics are able to permeate the
BTB. Thus enhancing metastases are different amongst each other. We are not the first to report heterogeneity in permeability of brain metastases. Zhang et al. used intravenous sodium fluorescein and histology to show that there was variability in the BBB permeability of brain metastases generated in mice from 8 different human cancer cell lines (breast, colon, renal and melanoma) (18). Lockman et al. demonstrated that breast cancer brain metastases in nude mice showed a range of values for passive permeability to the 3kDa tracer Texas red fluorescent dextran (16). While most metastases (~90%) showed increased permeability compared to normal brain, the degree of permeability of brain metastases varied widely and was much lower than that measured in peripheral tumours. This work was followed up by an in vivo MRI study conducted by Percy et al., in our lab (17). Percy et al. showed that no enhancing metastases were visualized at the early time point with T1wSE using Gd-DTPA. A wide range of volumes for enhancing and nonenhancing metastases was observed and it was also shown that metastasis volume and growth rates did not dictate whether or not a metastasis would become enhanced. In this thesis we have expanded on these findings by looking at brain metastases due to melanoma, and by including an additional imaging time point to improve the temporal sampling in vivo.

To understand why differences in permeability exist between metastases we first looked at whether their size or location in the brain was predictive of permeability. We found no relationship between location in the brain and enhancement after Gd-DTPA. Although the size of metastases increased with time we found no significant difference in the volumes, measured from MRI, for enhancing and nonenhancing metastases at any imaging time point. These findings are very similar to what was previously observed by Percy et al., for brain metastases due to breast cancer (17). Lockman et al. also reported no clear relationship between the diameter of brain metastases, measured by histology, and the degree of permeability to dextran (16). Our results, however, contradict other studies that have looked at the relationship between tumour size and BBB integrity since we observed no clear relationship between size and permeability.
An early study by Hasegawa et al. used phosphorescence imaging and radioactive alphaminoisobutyric acid (AIB) to show that the permeability of breast cancer brain metastases in the rat varied with the size of the tumor (62). Parenchymal tumors less than 1 mm in diameter showed no increased permeability to AIB. As the tumors enlarged over 1 mm in diameter, the permeability increased proportionally. Permeabilities of these brain metastases were less than one-third of the permeability of subcutaneously transplanted breast tumors. The results of Zhang et al., suggest that the permeability of the BTB is related to both the growth pattern/morphology and the size of the metastases. They showed that brain metastases were not permeable until they formed large masses (>0.2 mm); this was true for 2 different tumour morphologies; solid nodules and diffuse clusters of cells that eventually coalesced (18).

Historically, changes to the integrity of the BTB have been associated with large brain tumours (1-4 mm), which become oxygen and nutrient deprived and develop angiogenic vessels (63-65). Our data, however, clearly show that even very small metastases may be leaky. The differences between our results and those of others may be related to a number of factors including: differences in the specific tracer or contrast agent used, the cell lines and the timing of the interrogation of tumours. Together all of these studies reveal a complex relationship between metastasis size and permeability.

To investigate differences amongst enhancing and nonenhancing brain metastases, future studies may focus on examining molecular markers involved with maintenance of BBB integrity and function. Claudin-5, Occludin and ZO-1 could be tested in combination with endothelial markers such as CD31 and CD105 (endoglin) (66-69) to determine which tumours are undergoing angiogenesis. An interesting finding from the Lockman study was a relationship between overexpression of desmin pericyte protein, a component of the BTB. Other markers such as BRAF, STAT3, b-FGF, VEGF, GLUT-1 (70-73) may also be useful for molecular analysis to delineate differences between these types of metastases.
It has been demonstrated in other studies (19,29) that tumour vessel co-option does not cause disturbance to the endothelium of the BBB and the barrier remained intact as metastasis were not visible in contrast enhancement (CE) using Gd-DTPA. This might explain why some metastases in our study were nonenhancing. Two reasons that could explain the presence of enhancing metastases in our study are that 1) some could be secreting VEGF while remaining co-opted to pre-existing cerebral blood vessels and 2) some metastases remaining co-opted could be secreting VEGF and inducing angiogenesis. There is also the possibility that different vascularization processes such as vessel co-option, angiogenesis, and vasculogenic mimicry (74-76) are occurring simultaneously and may be influencing or controlling the transition to enhancement in a metastasis.

T1wSE with Gd-DTPA is the gold standard for brain tumour imaging in the clinic (58,77). Therefore, the fact that bSSFP detected nonenhancing metastases has important clinical implications. Our findings suggest that some metastases may go undetected by T1wSE with Gd-DTPA imaging. This warrants further study of bSSFP and its ability to detect small, nonpermeable metastases. We also observed that some melanoma brain metastases appeared as isointense in bSSFP, this presents as a problem since some metastases are missed as well in bSSFP images in the case of melanoma, due to its variable appearances in MRI. Future work by our lab will compare bSSFP to the conventional MRI pulse sequences in a clinical setting, to evaluate the possibility of improving early detection of brain metastases.

Brain metastases are an important clinical problem. Brain metastases contribute to death in nearly 95% of melanoma patients, with a median survival of less than 1 year despite treatment (2,78). There has been little improvement in this prognosis during the last 3 decades but new approaches are on the horizon. Preclinical models of metastatic disease provide a platform for the development and testing of new treatments and preclinical MRI is well suited to aid in the acceleration of these discoveries. The MRI methods used in this thesis to image brain metastases in mice is directly applicable to human studies. Future work in our lab will use this model to compare the in vivo response of BBB permeable and nonpermeable brain metastases to chemotherapeutics and radiation therapy. These
experiments will improve our understanding of the effects of treatment on tumour permeability and assist in the development of more effective treatments that could lead to better clinical management of patients with brain metastases.
2.5 References


27. Kaur J, Tuor UI, Zhao Z, Barber PA. Quantitative MRI reveals the elderly ischemic brain is susceptible to increased early blood-brain barrier permeability following tissue plasminogen activator related to claudin 5 and occludin disassembly. J Cereb Blood Flow Metab. 2011 Sep;31(9):1874-85.


64. Folkman J. What is the evidence that tumors are angiogenesis dependent. J Natl Cancer Inst. 1990 Jan 3;82(1):4-6.


Chapter 3

3 Study Implications and Future Directions

3.1 Summary of Key Findings

This study used a noninvasive magnetic resonance imaging approach to establish and characterize a clinically relevant mouse model of melanoma brain metastasis. To the best of our knowledge, this is the first study to characterize a clinically relevant model of melanoma brain metastasis using MRI. This model was then implemented in longitudinal MRI permeability studies. Using an established MRI-based protocol for detection of enhancing and nonenhancing brain metastases (1) we were able to monitor alterations in the blood-tumour barrier (BTB), and measure metastasis volume. Histological analysis was used to compare permeability findings with in vivo permeability findings and examine differences in metastasis morphology. Five key findings from this project are summarized below.

3.1.1 Characterized Model of Melanoma Brain Metastasis using MRI

An intracardiac injection of 50,000 human melanoma cells produced 5-19 brain metastases per animal. Meningeal and parenchymal metastases were detected, mimicking the human disease. In the clinically relevant model we have characterized, mice showed signs of neurological deficits such as hemiplegia (paralysis of one side of the body). Intracranial hemorrhage was apparent as well as the manifestation of hemorrhagic metastases (See Appendix E for MRI image and photographs showing hemorrhage). Hydrocephalus was also observed in the T1wSE and bSSFP MRI images.
3.1.2. Longitudinal Magnetic Resonance Imaging Studies: Early Detection of Melanoma Brain Metastases using bSSFP

Our main observation was that many metastases detected at the early time point (day 22) using bSSFP were nonenhancing, whereas only two metastases were shown as enhancing at the early time point (day 21) using T1wSE with Gd-DTPA. Interestingly, the number of metastases detected at the early time point using T1wSE with Gd-DTPA was never greater than one per animal. Most metastases detected at the early time point (day 22) were nonenhancing and many were small in volume ranging from 0.0247mm$^3$ to 0.0906mm$^3$.

3.1.3. Longitudinal Magnetic Resonance Imaging Studies: Variability in Enhancement and Patterns of Enhancement

Substantial heterogeneity in enhancement of brain metastases was observed throughout the study (Table 2.3.1 experiment 2; groups 1&2); each mouse had different numbers of enhancing metastases. Also, various patterns of enhancement were also observed (Chapter 2, figure 2.3.15) in different mice with melanoma brain metastases. Some metastases first appeared as nonenhancing and switched to enhancing at later time points; whereas, others that first appeared as enhancing remained enhancing throughout the experiment. There was one instance where a detected metastasis remained nonenhancing for the course of the study. Thus from these observations it would suggest that metastasis enhancement is a heterogeneous event with respect to the BTB.

3.1.4. Longitudinal Magnetic Resonance Imaging Studies: Volume, location and Age of Enhancing and Nonenhancing Brain Metastases

Using the MRI-based protocol established by Percy et al. (1) a range of volumes were measured for enhancing and nonenhancing metastases. We observed that there was no difference between the volume of enhancing and nonenhancing metastases at any time point. There was also no clear relationship between brain location and enhancement. In terms of age (when it was first detected to experimental endpoint) of the metastasis, metastases
detected for the first time at the late time point were nonenhancing and some were large and small in volume. Some metastases that were first detected as enhancing were also small in volume. From these longitudinal analyses we have observed that there is no direct correlation or relationship between volume, brain location or age of the metastasis and metastasis enhancement. To the best of our knowledge this is the first study using a noninvasive longitudinal imaging approach to investigate alterations of the BTB in melanoma brain metastases.

3.1.5 Histology: *Ex vivo* Permeability vs. *In vivo* Enhancement

We observed, in most cases, disagreement between the permeability of dextran and the contrast enhancement of metastases in T1wSE MRI images. For the nonenhancing metastasis, there were trace amounts of dextran leakage into the tumour interstitial regions, which we did not expect. This finding indicated that the BTB in certain regions of nonenhancing metastases is not completely intact. *Ex vivo* permeability analysis was performed to validate our *in vivo* MRI enhancement data. Dextran Texas Red was used as the *ex vivo* permeability tracer in this study.

3.2 Biological Explanations of Metastasis Enhancement

Biological factors speculated to be involved with alterations of the BTB include: 1) BTB modulation by vascular endothelial growth factor (VEGF)-A, 2) alteration in structure of the blood-brain barrier (BBB) and 3) enzymatic activation and cell-cell communication.

3.2.1 BTB Modulation by VEGF-A

VEGF-A is an important proangiogenic protein factor necessary for the promotion of angiogenesis. VEGF-A also functions to induce hyperpermeability and vasodilation in blood vessels (2-4). In the present study we did not evaluate which tumours were undergoing angiogenesis. Oliver *et al.* used A2058 human melanoma cells in, *in vitro* co-cultures and *in
animal studies to demonstrate that VEGF and interleukin-8 (IL-8), (another proangiogenic factor), were actively secreted (5). This indicates that this cell line has the potential to be angiogenic and may use angiogenesis as another means to obtain blood supply. In our study it is plausible that VEGF secretion might be involved with permeability changes of the BTB.

3.2.2 Alteration in Structure of the BBB

Pericytes function to control the permeability and integrity of the BBB and are also involved in communication via gap junctions (through which soluble factors transverse) with endothelial cells and other cells of the BBB (6,7). It has been shown that a loss of pericytes in the brain is associated with an increase in permeability (6,7). Astrocytes are similarly involved in regulating BBB integrity and studies have revealed that selective loss of astrocytes results in altered BBB function through changes in expression of tight junction (TJ) protein(s) (8). In our study the fact that some metastases changed to enhancing would suggest that astrocytic loss was prevalent. This warrants further investigation and could be evaluated using glial fibrillary acidic protein (GFAP) staining to confirm the presence or absence of astrocytes within or surrounding the BTB.

3.2.3 Enzymatic Activation and Cell-Cell Communication

Proteolytic enzymes are degradative enzymes secreted by cells to breakdown the local extracellular matrix (ECM) (9). These enzymes have been implicated in cancer metastasis during the intravasation process and in the initiation of BBB damage after extravasation (10). Fazakas et al. demonstrated that one of the mechanisms of transmigration for A2058 and B16F10 melanoma cells into the brain parenchyma was via the paracellular migration pathway (movement through interendothelial TJ) (11). The authors observed that gelatinotic serine protease secretion by the melanoma cells caused interendothelial TJ disruption as well as endothelial cell apoptosis (11). Interestingly it has been reported that the presence of matrix metalloproteinase 9 (MMP-9) is also involved with BBB impairment in brain trauma injury (12). MMP-9 expression by pericytes, astrocytes and cerebral endothelial cells
typically occurs during pathological conditions (6). This suggests that the secretion of various proteases and proteinases may play a role in the induction of damage to the BBB through degradation of TJs.

Astrocytes, perivascular macrophages, and neurons have been shown to be involved in BBB induction processes (13). In vitro studies have shown that astrocyte-endothelial cell interactions are important for mediation of unique characteristics towards the BBB (13). Astrocytes are known to secrete agents such as transforming growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF) which are important for the induction process (a cell-cell communication process) in which the endothelium becomes specialized (13). Estrada et al. and Mi et al. had demonstrated the importance of soluble factors produced from cerebral vessels for induction of growth and differentiation of astrocytes (14,15). This would suggest that during pathological conditions there is loss of normal barrier induction cell-cell communication signals from astrocyte to endothelium and the converse endothelium to astrocyte. Hence it is possible that in the (BTB) there are alterations in the induction process between astrocyte and endothelium and vice versa.

### 3.3 Impact of Key Findings

Early detection of brain metastases is important for proper patient management and treatment. The presence of nonenhancing brain metastases at early time points that would not be detected in current clinical imaging protocols (using T1wSE with Gd-DTPA) suggests the need for improved clinical imaging. Furthermore, current treatments rely on permeating the compromised BTB. However, Lockman et al. demonstrated that the extent of permeability for breast cancer brain metastasis varied (16). In our preclinical study we also showed that not every metastasis detected showed enhancement, and the timing of enhancement for the metastases was variable. Thus, the heterogeneity of enhancement should be considered in treatment planning because our assessment of brain metastases has shown that not all large metastases are necessarily permeable and some small metastases do show permeability. Another important point to consider is that even if a metastasis is permeable this does not
necessarily mean that a chemotherapeutic drug will be able to extensively permeate the BTB. This suggests the need for better therapeutic treatments that will be able to circumvent an intact BTB.

### 3.4 Study Limitations

In this study we have shown that MRI can be used to characterize a clinically relevant model of brain metastasis; there are however some limitations that should be addressed. First, the two MRI scans were performed on different days so there is the potential for a change in tumour characteristics in this time. This was done because Gd-DTPA takes 24 hrs to clear from the mouse circulation thus we have to wait for this clearance before imaging using bSSFP. From a clinical standpoint it would be more efficient and convenient to perform scans on the same day. Also the optimized T1wSE sequence that is used in our study uses 11 slices thus some enhancing metastases might be missed since only a certain portion of the mouse brain is covered. From a practical approach it would be worthwhile to increase the image slice numbers to allow for better coverage of the entire mouse brain. This however would mean that our scan time would be increased as well.

The sensitivity of our study was limited by image resolution. The highest resolution achieved by our MRI scans was 100-µm in plane. Thus we were not able to see smaller metastases i.e. micrometastases and therefore our MRI protocols underestimate the total number of metastases detected. Also, some metastases presented as isointense on bSSFP images and we did not count them because they were not visible. However, these metastases were visible in H&E histology, which would help to validate the presence of a metastasis.

Gadopentatic acid (Gd-DPTA) is used in the clinical setting and is typically administered by a bolus intravenous (IV) injection (17). In our model the intraperitoneal (IP) injection route was used because our T1wSE scans were 15 min and if we were to inject Gd-DTPA by the intravenous (IV) route the wash-in and washout times of Gd-DTPA are rapid 3min - 4min. Lastly, in this disease model it was difficult to maintain longevity of mice for longitudinal
studies since they were exhibiting disease onset by week 3 and most were dying at week 4, which is when the longitudinal studies would commence. Thus larger batches of mice are required for imaging during longitudinal studies of melanoma brain metastasis. Another option would be to include additional earlier time points in the longitudinal study.

3.5 Future Directions

Areas of interest for future investigations of this project include: histological characterization of enhancing and nonenhancing melanoma brain metastases, use of this model for testing chemotherapeutic potential or efficacy in treating brain metastases. And lastly, the use of bSSFP as an addendum clinical sequence for detection of nonenhancing metastases in the clinical setting, and using other future MRI studies for studying enhancing and nonenhancing brain metastases.

3.5.1 Histological and Immunohistochemical Characterization of Enhancing and Nonenhancing Melanoma Brain Metastases

An important area of future research for melanoma brain metastases is the immunohistochemical characterization of enhancing and nonenhancing melanoma brain metastases using angiogenic and BBB markers. By further investigating markers that are uniquely expressed and associated with enhancing and nonenhancing brain metastases, respectively, we will be able to gain a better understanding of their inherent differences. Ultimately we believe that this will help to propel better treatment planning in terms of drug development. Molecular factors that would be of interest for testing include BRAF which is involved with growth, survival, VEGFA and STAT3 which are involved with angiogenesis, and GLUT-1, which is involved with BBB development.

It would also be interesting to histologically evaluate tissue sections corresponding to contrast enhancing and nonenhancing brain metastases visualized by MRI at each time point.
This would permit us to compare tissue morphologies over time and examine whether subtle differences exist between tumour types that were not evident at the experimental endpoint.

3.5.2 Testing Therapeutic Efficacy using a Clinically Relevant Model

The goal of treatment, is to treat metastases as early as possible; however, the reality is that most brain metastases are detected at later stages of disease. The clinically relevant model we have established has the potential to be used in testing the efficacy of novel chemotherapeutic agents and in the evaluation of alternative methods for delivery of chemotherapeutic agents to target sites. Use of this model would aid tremendously in observing how melanoma brain metastases would respond to these therapies and potency of these drugs. Also there is the potential of this model to be used with other therapeutic modalities such as radiotherapy, radiosurgery, immunotherapy and targeted therapy (19,20). This model could also be used for different stages of disease onset. We are confident that this clinically relevant model of melanoma has the potential to be used in the evaluation of new treatment options.

We also aim to use this model to test the efficiency of alternative methods for drug delivery across the intact BBB. Some attractive proposed mechanisms include the use of neural stem/progenitor cells (NSPCs) loaded with 5-fluorocytosine (5-FC) pro-drug (inactive drug) of the chemotherapeutic agent and NSPCs loaded with the enzyme cytosine deaminase (CD) used to activate the pro-drug (21). Nanocarriers/nanoparticles such as poly (n-butyl cyanoacrylate) (PBCA) (22) and polyethylene glycol (PEG) liposomal nanocarriers (23) are being developed as vehicle agents for drug delivery. Focused ultrasound (FUS) guided MRI techniques have also been used as a method to temporarily disrupt the BBB (24,25). Our model could be used to test the delivery and efficacy of these new drug carrier methods.

3.5.3 Translation of the bSSFP Pulse Sequence for Brain Metastasis Detection in the Clinic

The goal of establishing a clinically relevant animal model is to be able to translate preclinical findings to the clinical setting. Currently no reports in the literature have shown
bSSFP used for the detection of human melanoma metastases in visceral organs or the brain. We have shown that nonenhancing metastases are detectable on bSSFP images at early time points. This reinforces the need to use alternative clinical sequences because brain metastases that do not enhance may be missed using current brain metastasis detection methods. Also another important point to mention is that amelanotic melanoma brain metastases may also appear isointense in T1w and T2w images as well as in bSSFP. Thus, if these metastases do not enhance using Gd-DTPA then they would most certainly be missed in brain metastasis detection. The implementation of bSSFP in clinical imaging protocols would increase the potential for earlier detection of nonenhancing metastases. However, this would only apply to metastases that do not appear isointense (in the case of melanoma brain metastases).

3.5.4. Future MRI Studies for Melanoma Brain Metastases

In future MRI studies we would like to use iron oxide contrast agents to evaluate the vascularity of enhancing and nonenhancing brain metastases. As a pilot project, we have imaged two mouse brains at day 14 and day 31 pre- and post-USPIO (Molday) contrast agent. Analysis of post-contrast imaging showed that blood vessels were present and some appeared to be dilated. (See Appendix F for bSSFP images with pre- and post-Molday contrast agent).
3.6 References


Appendices

Appendix A: Animal Use Protocol

The UNIVERSITY of WESTERN ONTARIO – UNIVERSITY COUNCIL ON ANIMAL CARE

ANIMAL USE PROTOCOL FORM - #2009-080 Chambers

A. INVESTIGATOR DECLARATION – Mandatory Completion

I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, “The Animals for Research Act,” of the Province of Ontario.

II. I confirm that this Animal Use Protocol accurately represents the proposed animal use.

III. I accept responsibility for procedures performed on animals in this project.

IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND be made familiar with the contents of this document.

1. I support the above declaration - YES ☒ Today’s Date mm/dd/yy:

B. PROTOCOL/INVESTIGATOR/FUNDING INFORMATION - Mandatory Completion

B.1. PROTOCOL INFORMATION

Application Type: Pick One Only. ☐ New ☑ Full Renewal ☐ Pilot ☐ Post Pilot Full Protocol

Protocol Title: Non-Invasive Imaging of Metastasis: Detection, Monitoring and Intervention

| Proposed Start Date: mm/dd/yy | 01/01/09 |
| Proposed End Date: mm/dd/yy | 06/30/10 |

If Full Renewal or Post Pilot Full Protocol:
Provide Previous Protocol #: 2005-052-09

If Full Renewal or Post Pilot Full Protocol, Provide a Progress Report Summary (Information taken directly from grant submission is not suitable): In the past year we have made progress on two fronts on this project: the molecular biology of brain metastases, and the methods for effective imaging of single metastatic cells and metastatic tumors using magnetic resonance imaging (MRI). In experimental studies, we have characterized the populations of cancer stem cells (CSCs) in two human breast cancer cell lines, and will now assess their abilities to form brain metastases. We also have characterized molecular pathways important for brain metastasis, including the Notch signaling pathway, which is important in CSC biology, and will extend this work to in vivo studies by inhibiting this pathway. We continue to optimize MRI imaging techniques to quantify two components of the cancer cell population in the brain – single dormant cells and growing metastases, and this work will continue during the upcoming year.

C. PROTOCOL OVERVIEW - Mandatory Completion

C.1. PURPOSE OF ANIMAL USE (PAU)

Pick the Primary Purpose of Animal Use (PAU): PAU 1 - Fundamental Research

Pick All Lesser Purpose of Animal Use (PAU) Elements:
☐ PAU 0 – Breeding Colony ☑ PAU 1 – Fundamental Research ☐ PAU 2 – Medical or Veterinary Research
☐ PAU 3 – Regulatory Testing ☐ PAU 4 – Drug/Product Development ☐ PAU 5 – Education or Training

C.2. SPECIES-SPECIFIC USE OVERVIEW

<table>
<thead>
<tr>
<th>Species Type</th>
<th>Use Type</th>
<th>Pick All That Apply</th>
<th>Highest Category of Invasiveness (C) per Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>☑ Chronic</td>
<td>☑ Acute</td>
<td>D</td>
</tr>
</tbody>
</table>

PROCEDURAL OVERVIEW CHECKLIST - Pick All That Apply Per Species COMPLETE REQUIRED ADDENDA: SECTION L

<table>
<thead>
<tr>
<th>Procedure Type</th>
<th>Addenda Completion Required</th>
<th>Procedure Type</th>
<th>Addenda Completion Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agent Use - Non-Hazards</td>
<td>1, 2 &amp; 9</td>
<td>☑ Dietary Manipulation</td>
<td>N/A</td>
</tr>
<tr>
<td>Agent Use - Hazardous</td>
<td>2</td>
<td>Geneally Altered Animals</td>
<td>6</td>
</tr>
<tr>
<td>Anaesthesia</td>
<td>3</td>
<td>Genotyping</td>
<td>N/A</td>
</tr>
<tr>
<td>Antibody Production</td>
<td>N/A</td>
<td>Hazards – Human &amp; Animal</td>
<td>9</td>
</tr>
<tr>
<td>Behavioural</td>
<td>4</td>
<td>Imaging</td>
<td>1, 2 &amp; 9</td>
</tr>
<tr>
<td>Blood Collection</td>
<td>5</td>
<td>Novel Model Creation</td>
<td>N/A</td>
</tr>
<tr>
<td>Breeding</td>
<td>N/A</td>
<td>Surgical Procedures</td>
<td>2</td>
</tr>
<tr>
<td>Collection - Other</td>
<td>N/A</td>
<td>Teaching Animals</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wildlife Field Study</td>
<td>8</td>
</tr>
</tbody>
</table>
Appendix B: Inter and Intra observer metastasis volume measurements

The reliability of metastasis volume measurements was determined by inter observer and intra observer volume measurement using the manual trace method. Agreement indices (see references Chapter 2 Filipi et al. and Joe et al.) and percentage differences were used to calculate the closeness in average metastatic volume measurements for the reference observer versus vs. outside observer as well as the reference observer vs. reference observer.

The percentage difference equation used was as follows:

\[
\frac{\text{larger number} - \text{smaller number}}{\text{smaller number}} \times 100
\]

The following equation was used to obtain the inter- and intra-observation reliability measurements (agreement indices) for metastasis volumes:

For inter-observation
larger number= observer 2 (outside observer)
smaller number= observer 1 (reference observer; myself)

For intra-observation
larger number= observer 1 (same individual; myself)
smaller number= observer 1 (same individual; myself)

The agreement index (AI) used (Filipi et al. Brain 1995) is as follows:

AI (agreement index) inter-observer agreement index = 1 - \frac{|X_a - X_b|}{(X_a + X_b)/2}

AI intra-observer agreement index= 1 - \frac{|X_{1st} - X_{2nd}|}{(X_{1st} + X_{2nd})/2}
The closer the agreement indices are to 1 the better the correlation.

Both inter- and intra-observer tumour metastasis measurements showed high agreement indices. Thus with confidence we can be assured that the measurements obtained for metastasis volumes in this thesis project are reliable measurements.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Percentage Difference (%)</th>
<th>Agreement Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>0.89</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>0.72</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Example of inter-observation metastasis volume measurements graphical correlation (Mouse ID 1, N=11 metastases)
Example of intra-observation metastasis volume measurements graphical correlation (Mouse ID 2, N=4 metastases)

We see that intra-observation provided good correlation with volume
Appendix C: Total metastatic burden

The data in this table shows the total metastatic burden for each mouse at different time points during the longitudinal study. Metastatic burden for each mouse increased over time.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Scan Days</th>
<th>Day 22</th>
<th>Day 25</th>
<th>Day 28</th>
<th>Day 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp1</td>
<td>1</td>
<td>18</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>12</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Exp2</td>
<td>5</td>
<td>6</td>
<td>13</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10</td>
<td>27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1</td>
<td>9</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>
Appendix D: Total, mean metastasis number, and mean volumes for meningeal and parenchymal brain metastasis

This table shows the total number of meningeal and parenchymal metastasis, the mean numbers and volumes for meningeal and parenchymal metastasis.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>cell dosage (X10³)</th>
<th># metastasis (total)</th>
<th># metastasis (meningeal)</th>
<th># metastasis (parenchymal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>8</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>19</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>19</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>13</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>13</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>12</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total (n=9)</strong></td>
<td></td>
<td><strong>102</strong></td>
<td><strong>22</strong></td>
<td><strong>81</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td><strong>11</strong></td>
<td><strong>2</strong></td>
<td><strong>9</strong></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>5.31</td>
<td>1.87</td>
<td>5.22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>#metastasis (meningeal)</th>
<th>#metastasis (parenchymal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume (mm³)</td>
<td>39</td>
<td>29</td>
</tr>
<tr>
<td>Mean volume (mm³)</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.52</td>
<td>1.18</td>
</tr>
</tbody>
</table>
Appendix E: Melanoma brain metastases hemorrhage

The following are examples of hemorrhage observed in the mouse brain from MRI image and removal of mouse brain after mouse sacrifice.

Clinically relevant model endpoint day 25: intracerebral hemorrhage observed in bSSFP image (A, yellow arrowhead) and H&E histology (B, black arrows), white arrows in B indicate presence of neutrophils. The nucleus of neutrophils is segmented (lobed nuclei) or band form (curved or half-moon appearance).

Longitudinal study endpoint day 31: Brain hemorrhage (C, D white arrows) for two different mouse brains.
Appendix F: Pilot study using USPIO (Molday) for tumour vasculature analysis

bSSFP image day 14: (pre and post Molday contrast agent injection)

Figure Appendix G: Shown are bSSFP images from different mice. (A&B) pre and post Molday bSSFP images of mouse brain at day 14; in (A) no metastases are visible at day 14. In (D) both enhancing metastases (yellow arrows) displayed differences in vascularization at day 31. Post molday injection (D) shows blood vessels containing molday label (white arrows).

Using bSSFP, pre and post Molday images were acquired. There was a noticeable difference in the pre and post images. In the post Molday images, brain vasculature as well as vasculature within the brain metastasis were seen as regions of hypointensity (loss of signal). We also wanted to look at patterns of vascularization of brain metastases using the Molday iron contrast agent at day 14 and day 31. At day 14 (A&B) no metastases were observed and only the brain vasculature was outlined with Molday contrast agent. It was observed that the vasculature of the metastases (D) was clearly outlined with the Molday as opposed to the pre Molday imaging. In (D) both of these metastases (yellow arrows) were found to be enhancing with Gd-DTPA at day 31, the blood vessels in the larger metastasis were much easier to see compared to the smaller metastasis at the top of the brain. Post Molday
intravenous (IV) injection showed intratumoral as well as peritumoral vasculature among some metastases (white arrows), also some of these vessels appeared to be larger and dilated compared to other metastases. Throughout this mouse brain, some metastases appeared to be more vascularized compared to others. Using Molday we were able to see more clearly the metastasis vasculature and more importantly the pattern of vascularization.

**bSSFP MRI parameters for pre and post Molday:**

FOV = 1.5, TR/TE=10/5 ms, FA= 35°, image matrix = 150 x 150, NEX= 4, rBW =±11.90 kHz, PC =4. The scan time was 38.25min and the spatial resolution was 100 x 100 x 200 μm.
Appendix G: Melanin images

The following H&E images (from the same mouse brain) show the presence of melanin pigmentation (A, yellow arrows) for a nonenhancing metastasis and an enhancing metastasis (B,C yellow arrows).
Appendix H: License and Permission usage

Nature Publishing Group License (Chapter 1)

<table>
<thead>
<tr>
<th>License Number</th>
<th>2938420142208</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Jun 29, 2012</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Nature Publishing Group</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Nature Reviews Cancer</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Metastasis: Dissemination and growth of cancer cells in metastatic sites</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Ann F. Chambers, Alan C. Groom and Ian C. MacDonald</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Aug 1, 2002</td>
</tr>
<tr>
<td>Volume number</td>
<td>2</td>
</tr>
<tr>
<td>Issue number</td>
<td>8</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Requestor type</td>
<td>academic/educational</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>High-res required</td>
<td>no</td>
</tr>
<tr>
<td>Figures</td>
<td>Figure 1 page 564 The metastatic Process (panels a and b)</td>
</tr>
<tr>
<td>Author of this NPG article</td>
<td>no</td>
</tr>
<tr>
<td>Your reference number</td>
<td>None</td>
</tr>
<tr>
<td>Title of your thesis / dissertation</td>
<td>In vivo MRI for assessing the integrity of the blood-tumour barrier in a mouse model of melanoma brain metastases</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Aug 2012</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>120</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.00 USD</strong></td>
</tr>
</tbody>
</table>
## Elsevier License (Chapter 1)

| Supplier            | Elsevier Limited
|---------------------|------------------|
|                     | The Boulevard, Langford Lane
| Registered Company Number | 1982084
| Customer name       | Mariama Henry
| Customer address    | Robarts Research Institute
|                     | London, ON N6A 5K8
| License number      | 2938410523194
| License date        | Jun 29, 2012
| Licensed content publisher | Elsevier
| Licensed content publication | Neurobiology of Disease
| Licensed content title | Structure and function of the blood–brain barrier
| Licensed content author | N. Joan Abbott, Adjanie A.K. Patabendige, Diana E.M. Dolman, Siti R. Yusof, David J. Begley
| Licensed content date | January 2010
| Licensed content volume number | 37
| Licensed content issue number | 1
| Number of pages     | 13
| Start Page          | 13
| End Page            | 25
| Type of Use         | reuse in a thesis/dissertation
| Portion             | figures/tables/illustrations
| Number of figures/tables/illustrations | 1
| Format              | both print and electronic
| Are you the author of this Elsevier article? | No
| Will you be translating? | No
| Order reference number | None
| Title of your thesis/dissertation | In vivo MRI for assessing the integrity of the blood-tumour barrier in a mouse model of melanoma brain metastases
| Expected completion | Aug 2012
| Estimated size (number of pages) | 120
| Elsevier VAT number | GB 494 6272 12
| Permissions price   | 0.00 USD
| VAT/Local Sales Tax  | 0.0 USD / 0.0 GBP
| **Total**            | **0.00 USD**
### Springer License (Chapter 1)

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>License Number</td>
<td>2938420850382</td>
</tr>
<tr>
<td>License date</td>
<td>Jun 29, 2012</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Springer</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Principles and applications of balanced SSFP techniques</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Klaus Scheffler</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Nov 1, 2003</td>
</tr>
<tr>
<td>Volume number</td>
<td>13</td>
</tr>
<tr>
<td>Issue number</td>
<td>11</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Thesis/Dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>Figures</td>
</tr>
<tr>
<td>Author of this Springer article</td>
<td>No</td>
</tr>
<tr>
<td>Order reference number</td>
<td>None</td>
</tr>
<tr>
<td>Title of your thesis / dissertation</td>
<td>In vivo MRI for assessing the integrity of the blood-tumour barrier in a mouse model of melanoma brain metastases</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Aug 2012</td>
</tr>
<tr>
<td>Estimated size(pages)</td>
<td>120</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>
Curriculum Vitae

Name: Mariama N. Henry

Post-secondary Education and Degrees:

University of Western Ontario
Windsor, Ontario, Canada
2010-2012 MSc. Medical Biophysics

Wayne State University
Detroit, Michigan
2003-2005 BSc. Clinical Laboratory Science (Magna Cum Laude)

University of Windsor
Windsor, Ontario, Canada
1999-2003 BSc. Honours Biochemistry with Thesis
Minor in Applied Information Technology

Honours and Awards

10th Annual Imaging Network of Ontario (IMNO) symposium, poster award. 2012, Toronto Ontario

Graduate Thesis Research Award Fund 2011, University of Western Ontario

University of Western Ontario- Schulich Graduate Scholarship 05/2011-08/2012

University of Western Ontario-Western Graduate Research Scholarship 09/2010-04/2011

Wayne State University, Dean’s List (4.0 GPA) 2004-2005

Wayne State University Medical Technology/Clinical Laboratory Science Alumni Association Scholarship 2004

American Society for Clinical Pathology (ASCP). National Student Honor award Wayne State University 2004

University of Windsor, Hutnik Undergraduate Research Award 2003
Related Work
Experience
Medical Laboratory Technologist
ASCP certified 2005-present
Henry Ford Hospital, Detroit, MI
2005-2008

After School Program Instructor
Mad Science of Windsor, Windsor, ON
2004-2005

Publications:
Conference Proceedings-Abstracts


