Role of CD15 and CD15s in the Cellular Mechanisms of Cancer Cell Metastasis from Lung to the Brain

Samah Ali Jassam

This thesis is submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the University of Portsmouth

January 2016
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DECLARATION

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not submitted for any other academic award.
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ABSTRACT

Non-small cell lung cancer is one of the most common primary tumours to metastasise to the brain in adults. The underlining molecular mechanisms of brain metastasis are still not fully understood. Interactions between brain endothelial cells and cancer cells play key roles in brain metastasis. CD15 and CD15s are cell-cell adhesion molecules which interact with E-selectin which is expressed on endothelial cells and known to be involved in the leukocyte homing process as well as being implicated in metastasis with many non-CNS neoplasms. The aim of this project was to investigate the role of CD15 and CD15s in cancer cell adhesion to brain endothelial cells and transendothelial migration of lung cancer cells during brain metastasis. Expression of CD15, CD15s and CD62E was characterised in human primary and brain metastatic lung cancer cells using immunocytochemistry, flow cytometry, Western blot and immunohistochemistry in human tissue sections. Effects of CD15 and CD15s expression on NSCLC metastatic to brain were assessed using genetic manipulation (overexpression and knockdown) followed by functional assays. Both CD15 and CD15s were overexpressed and knockdowned and cell-cell adhesion was then examined using qualitative and quantitative adhesion assays, under both static and flow physiological conditions. Transendothelial migration potential was also assessed using a voltmeter, Electric Cell-Substrate Impedance sensing system and cell-monitoring system CellZscope™. Findings showed that CD15 and CD15s were prominently expressed on metastatic lung cancer cells (SEBTA-001, SEBTA-005 and NCI-H1299) and weakly expressed on both primary lung cancer cells (COR-L105 and A549) and brain endothelium (hCMEC/D3). The highest expression of CD62E was observed on brain endothelium stimulated with TNF-α (25pg/ml) (p<0.001). CD15, CD15s and CD62E expression was detected in human metastatic tissues. The absence of CD62E and immunoblocking and knockdown of CD15 and CD15s significantly reduced the adhesion of cancer cells under both static and shear stress conditions (p<0.0001).
Overexpression of CD15 and CD15s significantly increased their adhesion on an endothelial monolayer (p<0.001). Metastatic cancer cells were able to transmigrate through a brain endothelial monolayer compared to primary and glioblastoma multiforme (GBM) cells. Knockdown of CD15 and CD15s decreased the transendothelial migration potential of cancer cells while even primary lung cancer cells and GBM cells transmigrated following overexpression of CD15 and CD15s. These results confirmed the correlation between CD15 and CD15s in adhesion as well as transendothelial migration of cancer cells during cerebral metastasis.

*Keywords: Metastasis, CD15, CD15s, E-selectin, adhesion, transendothelial migration*
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<tr>
<td>ALCAM</td>
<td>Activated Leukocyte Adhesion Molecule</td>
</tr>
<tr>
<td>ADAMs</td>
<td>A disintegrin and Metalloproteinase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>BM</td>
<td>Brain Metastases</td>
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<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecules</td>
</tr>
<tr>
<td>CCL2</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<tr>
<td>CT</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating Cancer Cells</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionised Water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOI</td>
<td>Diffuses Optical Imaging</td>
</tr>
<tr>
<td>ECIS</td>
<td>Electric Cell Impedance Substrate</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>EREG</td>
<td>Epiregulin</td>
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<td>EVOM</td>
<td>Voltohmmeter</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>FFPE</td>
<td>Formalin Fixed Tissue Sections</td>
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<td>GBM</td>
<td>Glioblastoma Multiforme</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<td>HRP</td>
<td>Horseradish Peroxide</td>
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<tr>
<td>HS</td>
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<td>HSPGs</td>
<td>Heparansulfate Proteoglycans</td>
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<td>IASLC</td>
<td>International Association for the Study of Lung Cancer</td>
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<td>mAb</td>
<td>Monoclonal Antibody</td>
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<td>MARK</td>
<td>Mitogen-activated Protein Kinases</td>
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<td>MMP-2</td>
<td>Matrix Metalloproteinase-2</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NaCl₂</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa (Light-Chain-Enhancer of Activated B cells)</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Clinical Excellence</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
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<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Prophylactic Cranial Irradiation</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDT</td>
<td>Population Doubling Time</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>PSGL-1</td>
<td>P-Selectin Glycoprotein Ligand-1</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time-Polymerase Chain Reaction</td>
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<tr>
<td>RCC</td>
<td>Renal Cell Carcinoma</td>
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<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis</td>
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<td>SRS</td>
<td>Stereotactic radiosurgery</td>
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<td>TBST</td>
<td>Tris-buffered Saline-Tween</td>
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<td>TEER</td>
<td>Trans-endothelial migration</td>
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<td>Trans-epithelial migration</td>
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<td>TGFβ1</td>
<td>Transforming Growth Factor Beta 1</td>
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<td>Tumour Necrosis Factor-Beta</td>
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<td>TNM</td>
<td>Tumour, Lymph node, Metastasis staging system</td>
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<td>Western blot</td>
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<td>World Health Organisation</td>
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<td>WBRT</td>
<td>Whole Brain Radiotherapy</td>
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Conferences and meetings

   Meeting: 1st year PhD student conference, Faculty of Science
   Location: University of Portsmouth, UK (May 2012).
   Poster presentation

2. Samah A Jassam, Qian An, Helen L Fillmore and Geoffrey J Pilkington.
   Meeting: The 10th Hammer out brain tumour patient conference.
   Location: London, UK (March 2013).
   Poster presentation

   Meeting: The 6th International Summer School- ISS2013-PartA
   Location: Piran, Slovenia (April 2013).
   Oral presentation

   Meeting: The Institute of Biomedical and Biomolecular Sciences (IBBS) postgraduate research day
   Location: University of Portsmouth, UK (May 2013).
   Poster presentation

   Meeting: The conference of Iraqi students at the University of Plymouth
   Location: Plymouth, UK (March 2014).
   Poster presentation

   Meeting: The Institute of Biomedical and Biomolecular Sciences (IBBS) postgraduate research day
   Location: University of Portsmouth, UK (May 2014).
   Oral presentation
*Meeting*: Metastatic disease meeting  
*Poster presentation*  

*Meeting*: The 9th International Conference of Anticancer Research  
*Location*: Sithonia, Greece (October 2014).  
*Oral presentation*  

*Meeting*: Early career UK and Ireland blood-brain barrier symposium  
*Poster presentation*  

*Meeting*: Early career UK and Ireland blood-brain barrier symposium  
*Oral presentation*  

11. Samah A Jassam, Zaynah Maherally, Helen L Fillmore and Geoffrey J Pilkington  
*Meeting*: The annual scientific meeting of British Society of Neuro-Oncology (BNOS)  
*Location*: Nottingham (July 2015).  
*Oral presentation*
*Meeting*: The annual scientific meeting of British Society of Neuro-Oncology (BNOS)  
*Location*: Nottingham (July 2015).  
*Poster presentation*

*Meeting*: The 20th annual scientific meeting and education day of the Society for Neuro-Oncology (SNO)  
*Location*: San Antonio, USA (November 2015).  
*ePoster presentation*

*Meeting*: IBBC 10th London Conference “Iraq in the Global Marketplace”  
*Oral presentation and panellist*

*Location*: Southampton, UK (November 2015).  
*Oral presentation*

*Poster presentation*
Publications

a. Abstracts

1-CD15 and E-selectin mediation of adhesion of non-small cell lung cancer cells to brain endothelium in lung-brain metastasis.
Authors: Geoffrey J Pilkington, Samah A Jassam, Zaynah Maherally, James Smith and Helen L Fillmore
Status: Abstract published

2-An all human 3D in vitro model of the blood brain barrier in nanoparticle delivery and cancer metastasis studies.
Authors: Pilkington GJ, Maherally Z, Jassam SA, Barbu E and Fillmore HL.
Status: Abstract published

3-CD15 and E-selectin mediation of adhesion of non-small cell lung cancer cells to brain endothelium.
Authors: Jassam SA, Maherally Z, Smith JR, Ashkan K, Fillmore HL and Pilkington GJ
Status: Abstract published

4-Expression of CD15 and CD15s is correlated with glioma cell arrest at G1-phase.
Authors: Samah A. Jassam, Zaynah Maherally, Paraskevi Charta, Keyoumars Ashkan, Helen L. Fillmore and Geoffrey J. Pilkington
Status: Abstract published

Authors: Jassam SA, Maherally Z, Smith JR, Ashkan K, Filmore HL and Pilkington GJ.


Status: Abstract published

b. Published papers

1- TNF-α enhancement of CD62E mediates adhesion of non-small cell lung cancer cells to brain endothelium via CD15 in lung-brain metastasis.

Authors: Jassam SA, Maherally Z, Smith JR, Ashkan K, Roncaroli F, Filmore HL and Pilkington GJ.


Status: Published

2- Expression of CD15 and CD15s is correlated with cell cycle arrest at G1 phase in primary and metastatic brain tumour cells.

Authors: Samah A. Jassam, Zaynah Maherally, Paraskevi Chairta, Helen L. Filmore and Geoffrey J. Pilkington.

Journal: Neuro-Oncology.

Status: Submitted

3- Transmigration of non-small cell lung cancer cells through the blood brain barrier: Role of CD15 and CD15s in metastasis from lung to the brain.


Journal: Cancer Research.

Status: Submitted
4- NSCLC cell adhesion to and transmigration through brain endothelium in brain metastasis.

Authors: Samah A. Jassam, Zaynah Maherally, Helen L Fillmore and Geoffrey J. Pilkington.


Status: Submitted

5- Interrupting the interaction between CD15s and E-selectin reduced the adhesion of non-small cell lung cancer cells to brain endothelial cells.

Authors: Samah A. Jassam, Zaynah Maherally, Helen L Fillmore and Geoffrey J. Pilkington.

Journal: Plos One.

Status: Submitted
## Laboratory work Contribution

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Last but not least, sincere thanks to the Merciful God for giving me the strength to carry on and lightening the darkness throughout the hard times.
DEDICATION

I dedicate this thesis to

Iraq and UK,

My mother, father, and beloved family,

to all my teachers and supervisors,

for all your unconditional and continuous love, care and encouragement.

You have been and will always be my strength and inspiration.
CHAPTER ONE

INTRODUCTION
1.1 Lewis Antigens

Lewis antigens are cell membrane carbohydrates. They can be categorised into two major types depending on their chemical structure. Lewis antigens-Type 1 include Lewis a (Galβ1,3(Fucα1,4)GlcNAc)(Leα(a)) and Lewis b (Fucα1,2Galβ1,3(Fucα1,4)GlcNAc)(Leβ(b)) (Cummings 2008). The antibodies against these carbohydrate epitopes are used in clinical detection of blood groups. Both Lewis a and Lewis b are expressed on the surface of red blood corpuscles (RBCs), Reed-Sternberg cells (Zukerberg et al., 1991) and many types of endothelial cells. Both are secreted in body fluids such as blood serum and saliva (Kabat, 1956). Lewis a is centrally involved in microbial adhesion and it is expressed on many types of pathogenic bacteria (Greenwell 1997, Peek and Blaser 2002, Holgersson and Lofling 2006 and Liu et al., 2009). Lewis b is expressed in many types of pathogenic bacteria but interestingly it does not play a role in microbial cell attachment (Zheng et al., 2003). Overexpression of Lewis “a” has been reported to be correlated with the malignancy of colorectal cancer cells (Narimatsu et al., 1996 and Muinelo-Romay et al., 2010). Lewis antigens-Type 2 include Lewis x (CD15) which will be discussed in section (1.4), and Lewis y (CD174) (Fucα1,2Galβ1,4(Fucα1,3)GlcNAc)(Leγ) (Muinelo-Romay et al., 2010). Many studies refer to the correlation of Lewis y and cell apoptosis (Baldus et al., 2006 and Wang et al., 2003). There is a positive correlation between Lewis y and cell dysplasia in colorectal adenomas and most adenocarcinomas (Abe et al., 1986, Kim et al., 1986, Waldock, et al., 1989, Baldus et al., 1995, 2002 and 2006).
1.2 CD15

CD15, Lewis x (Le\(^x\)) antigen is also known as stage specific embryonic antigen-1 (SSEA-1) (Figure 1). It is a trisaccharide, cell-cell adhesion molecule (Gooi et al., 1981 and Elola et al., 2007). It is expressed on different types of glycoconjugates at the surface of different cells. CD15 plays a key role in cell-cell interaction and communication during specific developmental stages as in the 8-cell to blastocyst stages, primordial germ cells, non-differentiated embryonic stem cells and embryonic carcinoma cells in mouse embryos (Kudo et al., 2004). Also, CD15-CD15 homophilic interaction is involved in cell differentiation and developmental process (Eggens et al., 1989). Moreover, its overexpression is associated with human polymorphonuclear granulocytes and various tumour cells such as lung, colon and breast carcinomas (Spooncer et al., 1984 and Mordoh et al., 1994). In the CNS, it is expressed in cortical neuroepithelial cells as well as in the radial glia cells of human embryonic brain (Hennen et al., 2011). It is reported that CD15 is not generally expressed on human glioblastoma multiforme (GBM) cells and anaplastic astrocytes (Martin et al., 1995). However, some studies revealed that CD15 is expressed on specific cancer cell population (Pruszak et al., 2007 and Pruszak et al., 2009). Furthermore, CD15 is localised on stem cell-like cells in human glioma and tumour spheres derived from murine astrocytoma and ependymoma (Mao et al., 2009). Recently, CD15 is considered a distinctive marker of cancer stem cells (CSCs) in medulloblastoma cells (Gate et al., 2015). It is also reported that CD15/CD24/CD29 represents “a marker profile that defines the lineage development of neural stem cell” (Pruszak et al., 2007 and Pruszak et al., 2009). It is also reported that CD15 is may be considered as a marker of tumour-propagating cell (TPC) (Read et al., 2009). In cancer metastasis, elevated expression of CD15 is correlated with the adhesion of some cancer cells during the metastasis process since CD15 has the ability to bind by homophilic interaction with CD15 expressed on endothelial cells and it initiates a heterophilic interaction with other cell adhesion molecules such as the selectins (Brooks and Leathem, 1995).
1.3 CD15s (Sialyl-CD15)

CD15s (SLe\(^\alpha\)) also called Sialyl Lewis x, is a tetra-polysaccharide molecule (Figure 2), expressed on the terminus of glycolipids of different cells. CD15s is also a ligand for E-selectin and P-selectin (Polley et al., 1991) and is involved in adhesion of cancer cells to endothelial cells (Vestweber and Blanks 1998, Giavazzi et al., 1993, Kitayama et al., 2000; Burdick et al., 2003). It mediates the initial steps of tethering and rolling of white blood cells during WBC homing process. The circulating cells head to the site of injury by initial binding between CD15s and E-selectin (Munro 1993; Aruffo and Glycosci 1994). Burdick et al., (2003) highlighted that metastatic colon adenocarcinoma cells adhere to human umbilical vein endothelial cells (HUVEC), through CD15s-glycolipids and CD15s-glycoproteins which bind to E-selectin. Wang et al., (2003) reported that CD15, CD15s and Sialyl dimeric CD15 (SDLe\(^\alpha\)) expression is associated with malignancy and reported their overexpression in human non-small pulmonary cancer (NSCPC) and human primary liver cancer (PLC) tissue while they are not expressed in the adjacent non-malignant tissue. Moreover, Ikeda et al.,(1996) showed that CD15s is expressed in 60% of patients with primary lesions of gastric cancer and in 51% of patients with advanced primary gastric tumour and metastatic lymph nodes. It is further reported that expression of CD15s may be used as a marker for the early diagnosis of cervical cancer (Nakagoe et al., 2002). Very little is however, known about CD15s in the CNS but we have recently reported that overexpression of CD15s is seen to be correlated with cell arrest in G1 phase of cell cycle in GBM and metastatic lung to brain cancer cells (Jassam et al., 2015b).
Figure 1: Structure of CD15 (Lewis X) 3-fucosyl-N-acetyl-lactosamine. CD15 is a tri-saccharide molecule consisting of three fucoses bound to N-acetyl-lactosamine by α1, 3 bonds. Figure adopted from Moore and Auzanneau 2012.

Figure 2: Structure of CD15s (sialyl-Lewis X) 3-fucosyl-N-acetyl-lactosamine. CD15s is a tetra-saccharide molecule consisting of three fucoses bound to N-acetyl-lactosamine molecule by α1,3 bond. Figure obtained from Moore and Auzanneau 2012.
1.4 Biosynthesis of CD15 and CD15s

Lewis antigens are encoded by the FUT gene family. These genes are distributed on the short and long arms of chromosome 19 and encode for fucosyltransferases enzymes (FUT) (Vries et al., 2001). FUT enzymes have the ability to transfer fucose sugar molecules in α1,2, α1,3/4 and α1,6 linkages close O-peptidic and N-peptidic sites on a wide range of glycan molecules (Javaud et al., 2003). Up to date, eleven FUTs are known, all are involved directly or indirectly in the synthesis of lewis antigens (Easton et al., 1993, Ikeda et al., 1996) as shown in Figure 3. The molecular mechanisms that regulate glycosylation process are not fully resolved; however, it has been shown that FUTs expression can be influenced by some cytokines as TNF-α which is shown to increase the expression of FUT3 and FUT4 but not FUT2. Also, IL-6 and IL-8 induction significantly increased the expression of FUT3 and FUT11 in bronchial mucins from patients with cystic fibrosis (Groux-Degroote et al., 2008). The molecular mechanisms that regulate the expression of CD15 and CD15s are still not fully understood and many mechanisms were proposed in different types of cells. Most studies focus on the synthesis of the fucosyltransferase that is located within the endoplasmic reticulum and Golgi apparatus which affect the expression of CD15 and CD15s (Kannagi 2001). The extracellular exposure of these epitopes is often attributed to the upregulation of these specific transferases (Nakayama et al., 2001). Groux-Degroote et al., (2008) revealed that expression levels of FUT3, FUT11 and CD15s are correlated in human bronchial mucins. In another study, CD15 and CD15s expression is seen to be regulated by Sialidase activity in human myloid cells (Gadhoum and Sackstein, 2008).
Figure 3: Schematic diagram shows the possible pathways of biosynthesis of CD15 and CD15s. The process occurs in the Golgi apparatus. Fucose molecules are added to a proteoglycan (O-glycan) molecule by fucosyltransferases (FUTs) and sialidase enzymes.
1.5 CD62E (E-selectin)

CD62E or E-selectin (E-LE) is a glycoprotein and cell-cell adhesion molecule (CAM), it is also named as an leukocyte-endothelial cell adhesion molecule 2 (LEAM-2) or endothelial-leukocyte adhesion molecule 1( ELAM-1). It is a member of selectins family, which includes other two members: CD62P and CD62L. All selectins are classified as a type 1-membran protein and C-type cell surface lectiens. CD62E is expressed on the cell surface of activated endothelial cells, in response to specific cytokines stimuli (Zukerberg et al., 1991 and Read et al., 2009). CD62E is composed of an N-terminal, a C-type lectin domain, an epidermal-growth factor like domain (EGP), 6 Sushi domains, a transmembrane domain and a cytoplasmic tail (Figure 4) (Graves et al., 1994). CD62E in human cells is encoded and regulated by the \textit{SELE} gene that is located on the chromosom 1; each domain of CD62E is encoded by one independent exon, however the cytosolic domain is derived from two exons (Cumming et al., 2008).

![Figure 4: Structure of CD62E](image)

Figure 4: Structure of CD62E. A Ribbon representation of CD62E structure showing CD62E protein chanes (gray), the bounded calcium ions (Ca$^{2+}$) represented by red sphere, fucose (green), galactose (yellow), N-acetyleuraminic acid (violet) and β-methyl-N-acetyl-D-glucosamine(blue). Modified figure obtained from Graves et al., 1994 and Protein Data Bank ID:1G1T.
CD62E extracellular expression is stimulated with specific cytokines such as: interleukin-1 (IL-1β) (Huang RB and Eniola-Adefeso 2012), lipopolysaccharide (LPS) (Dixon et al., 2004) and tumour necrosis factor (TNF-α). Also, it is suggested that CD62E formation can be activated in response to shear stress (Yoshizumi et al., 2003). The expression of CD62E is controlled by the activity of transcription factor NF-κB, which regulates the transcription process of SELE gene that encoding CD62E as in Figure 5 (Cernuda-Morollon and Ridley, 2006).

Figure 5: Regulation of CD62E extracellular expression

Figure 5: Schematic of regulation of CD62E expression. TNF-α, one of the important extracellular stimuli of CD62E expression, induces the activation of Rho GTPases which, in turn, promotes the transcription and translation of CD62E glycoprotein. Modified illustration adapted from Cernuda-Morollon and Ridley, 2006.
The molecular bases of CD62DE interactions are not fully unveiled. However, the discovery of CD62E crystal structure lectin/EGF explains the binding between CD62E and sialyl Lewis x (SLex)-type glycans (Graves et al., 1994). Previous studies showed that, CD62E has the ability to bind with specific fucosylated and sialylated carbohydrate epitopes which are present on glycan arms such as; CD15, CD15s and Lewis a which are expressed on neutrophils, monocytes, eosinophils and natural killer cells (NK) during inflammation and on many cancer cells types (Graves et al., 1994 and Somers et al., 2000). CD44, CD107a and CD107b are considered as CD62E ligands related to the sialylated carbohydrate epitope as sialyl Lewis x, y and a (Tomlinson et al., 2000, Gout et al., 2006, Napier et al., 2007).

CD62E plays an important role in the leukocyte tethering and rolling process through sugar-sugar interaction (Figure 6). Crystallography studies of CD62E and its interaction with the different ligands show there is a high affinity between CD62E and CD15s (Somers et al., 2000). CD62E was also shown to play an important role in the neutrophil rolling process both in vivo using a mouse model and in vitro using flow chambers. It has also been hypothesised that CD62P (P-selectin) and CD62E interactions with glycoconjugates expressed by leukocytes mediate the tethering process during inflammation (Zarbock et al., 2011). CD15/CD15s and CD62E interaction plays an important role in cancer cell metastasis, specifically during the intravasation and extravasation process of circulating cancer cells (Figure 6) (Laferriere et al., 2001). It is reported that CD62E is over-expressed on endothelial cells that are involved in homing the circulating cancer cells during formation of secondary discrete lesions in the lung (Hiratsukaa et al., 2011). Moreover, a neutralising CD62E via monoclonal anti-CD62E reduced the transendothelial migration in vivo of spontaneous murine glioma (SMA-560) cells.
Figure 6: Stereo view of CD62E (green) bound with fucosilated ligand (CD15s) epitop (purple). (A) The image is showing the interaction of fucose. (B) The image shows the interaction between galactose and NeuAc. A yellow sphere represents Ca$^{2+}$, H$_2$O bound molecules represented by red sphere and dashed lines are showing the hydrogen bonds and Ca$^{2+}$ ligation. The figures are obtained from Somers et al., 2000.
1.6 Role of CD15, CD15s and CD62E in brain metastasis

Distant secondary tumours originate from a single metastatic cell, which then migrate via vascular circulation (Al-Mehdi et al., 2000 and Narimatsu, et al., 2005). The mechanism of how metastatic cancer cells transmigrate through the vessels to target tissue is not fully understood. Both CD15 and CD15s have been regarded as tumour-associated markers as well as natural ligands of CD62E (Martin et al., 1995 and Soejim & Koda, 2005). Some cancer cells tend to use the same mechanism as leukocytes during extravasation suggesting that CD15/CD15s expressed on the surface of metastatic cells homophilicly interact with CD62E exposed on endothelial cells (Figure 7). It was therefore speculated that this process may explain why some cancer cells are more metastatic than others (Jassam et al., 2015). However, our knowledge about the role of CD15/CD15s and CD62E interaction and its ability of cells to cross the intact endothelial cells monolayer is still in its infancy and the molecular mechanisms underlying transmigration through the BBB remains unexplored.

Figure 7: Role of CD15, CD15s and CD62E in transendothelial migration

Figure 7: Schematic diagram depicting the potential role of CD15, CD15s and CD62E in trans-endothelial migration to the brain. Circulating cancer cells expressing CD15 or CD15s are able to adhere to activated endothelial cells expressing CD62E, induced by TNF-α or IL-1β, IL-6.
1.7 Cell-Cell adhesion

Cell-cell adhesion is an important process in cell coordination, communication and migration in multicellular organisms. It is a selective process achieved by interaction between specific cell adhesion molecules. There are several types of cell-cell adhesion molecules, which are classified into many major sub-groups according to their structural and functional characteristics such as selectins which include three highly specialised lectins (carbohydrate-binding proteins): P-selectin (CD62P), L-selectin (CD62L) and E-selectin (CD62E). All selectins are able to bind selectively with oligosaccharides, each of the selectins has a N-terminal domain (Ca\(^{2+}\)-dependent, C-type), an epidermal growth factor like motif, a transmembrane domain and a cytoplasmic tail (Bathel et al., 2007). The results of molecular modelling studies revealed that P and L-selectins have a similar structure while, E-selectin differs (Mcever 2001). Selectins play a key role in the homing process of circulating leukocytes to endothelial cells. The formation of weak and transient bonds is a key step called tethering. This is followed by rolling in which leukocytes form stronger bonds on their leading edge. In both tethering and rolling the binding is transient and a rolling cell may return to the circulation at any time or fully adhere to the endothelial cell. Selectins are reported to be involved in all the three steps (tethering, rolling and adhesion) (Schon and Ludwig, 2005). Other important cell-cell adhesion molecules are within the immunoglobulin superfamily (IgCAMs); these molecules function as cell adhesion molecules and signalling receptors. They play important roles in signal transduction from the extracellular to the intracellular compartment (Brummendorf and Rathjen 1995). It is revealed that IgCAMs are expressed in different tissues and play important roles in cell-cell and cell-ECM communication. IgCAMs are also involved in cell migration, induction of the neurite, plasticity and path finding processes during neural wiring at the embryonic stages (Walsh and Doherty, 1997). The IgCAMs Ig-like domain is considered to be the major structural feature of all IgCAMs and the wide range of diversity
in IgCAMs is reported to be related to the post-transitional modification or alternative splicing (Walsh and Doherty, 1997). The intracellular domain of IgCAMs interacts with many cytoplasmic functions and it has been suggested that it is involved in cell motility as it connects between the IgCAM cytoplasmic domain and the cortical cytoskeleton like ankyrins. IgCAMs potentially bind with other IgCAMs as well as binding with a variety of different proteins, forming important functional complexes such as, the interactions of axon-ECM, axon-axon and axon-glia (Volkmer 2001). It has also been demonstrated that IgCAMs are involved in signal transduction pathways and are associated with receptor and non-receptor tyrosine kinases, serine/threonine kinases and receptor tyrosine phosphatase (RPTP) (Volkmer 2001).

Cadherins are integral transmembrane glycoproteins, calcium-dependent cell-cell adhesion molecules (approximately 350 cadherins) (Hulpiau and Van-roy, 2009). This superfamily is categorised into four major sub-groups, which are: classical cadherins, protocadherins, desmosomal cadherins and cadherin-related proteins. All cadherins have a distinctive common structural feature in their extracellular compartment becoming multiple cadherin-specific repeats (CR) (Hulpiau and Van-roy, 2009). The main function of cadherins is to initiate a strong cell-cell adhesion such as the establishment of tight junctions between endothelial cells (Kishikawa et al., 2008). Cadherins are also involved in determining the selectivity and connectivity of neurons during the development of the central nervous system (CNS) (Radice and Takeichi, 2001). Other important cell-cell adhesion proteins are the integrin family which are transmembrane glycoproteins and cell-ECM adhesion molecules. They act as receptors for specific proteins in the ECM and have an important role in communication between the cell and the extracellular microenvironment. All integrins are heterodimer molecules composed of two glycoprotein units (α and β), each unit of which has the ability to interact with a wide range of cytoplasmic (intracellular) and microenvironment (extracellular) proteins (Dzamba et al., 2001). It is also reported that integrins are involved in cell cycle progression (Moreno-Layseca and Streuli 2014), cell survival (Vachon 2011), cell migration (Hood and Cheresh,
Heparan sulfate proteoglycans (HSPGs) are also cell adhesion glycoproteins which are categorized according to their location and are classified into three subgroups: the first subgroup includes the cell membrane HSPGs, glypicans and syndecans, the second subgroup includes secreted HSPG to the extracellular matrix such as agrin, collagen (type XVIII) and perlecanc and the third subgroup includes the secretory vesicle proteoglycan such as: serglycin (Sarrazin et al., 2011). All HSPGs are recognised by a common structural feature which is the existence of heparan sulfate chain (HS) (Sarrazin et al., 2011); HSPGs molecules interact with different ligands to control and initiate many important cellular activities such as cell-ECM adhesion and invasion (Lim et al., 2015). ADAMs (a disintegrin and metalloproteinase) are a group of transmembrane proteins expressed on the cell surface and secreted to the microenvironment of the surrounding tissue (Edwards et al., 2008). ADAMs have a distinguishing structure feature, the existence of an integrin receptor-binding, a metalloprotease and a cytoplasmic domain (Seals and Courtneidge, 2003). It is reported that ADAMs are involved in many important cellular activates such as, controlling the fusion process of cell membranes (Primakoff and Myles 2007), secretion of cytokines, cell migration and invasion (Stamenkovic 2000).
1.8 Cell transendothelial migration (TEEM) (diapedesis)

Transendothelial migration or diapedesis is the process where circulating cells migrate through the endothelial barrier in which cells leave the circulatory system towards surrounding tissue. Different leukocytes such as natural killer (NK), neutrophil, T-lymphocytes and monocytes are induced to transmigrate out of the circulation by inflammatory reaction via the release of specific chemokines (Muller 2011). Leukocytes leave the circulation through two different mechanisms: paracellular transmigration in which emigrant cells find their way out by amoeboid movement between the borders of tightly connected endothelial cells and transcellular transmigration in which emigrant cells migrate through the endothelial cells themselves (Muller 2013). Transmigration is not a random process, it only occurs if there is an inflammatory response which is caused by the secretion of histamine and other mediators which induce the release of P-selectin from Weibel-Palade bodies in the endothelial cytoplasm and express it on the surface of the endothelial cells. This, in turn, induces the extracellular expression of E-selectin (McEver and Cummings, 1997). Selectins have an affinity to bind with members of the Lewis blood group family that are expressed on leukocytes (Munro et al., 1992) and on some cancer cells (Martin et al., 1995 and Jassam et al., 2015). Extravasation of leukocytes or circulating cancer cells starts with tethering and rolling, followed by formation of firm adhesion and transmigration (Muller 2011). It is an interactive process: migrating cells, express different types of cell-cell adhesion molecules which interact with adhesion molecules expressed on endothelial cells. Extravasation of leukocytes is strictly controlled by secreted cytokines. It is hypothesised that circulating cancer cells tend to mimic the physiology of leukocytes during extravasation (Strell and Entschladen, 2008) but the molecular machinery beneath the extravasation of cancer cell differs from the one in leukocytes (Miles et al., 2008). E-selectin is a key element in homing circulating cancer cells in various cancers where the cancer cells are attracted to endothelial cells that express
a high level of E-selectin (Burdick et al., 2006). In a study on colon cancer cells, CD44, which is a ligand of selectins, was found to be upregulated and involved in mediating the rolling of cancer cells (Hanley et al., 2006). In colon cancer cells, both E-selectin and its ligand (PSGL-1) are overexpressed in prostate cancer metastases which are involved in metastasis of prostate cancer to the bones (Dimitroff et al., 2005). Sialyl lewis x, another E-selectin ligand has also been reported to mediate metastasis in breast cancer (Julien et al., 2011).

1.8.1 Paracellular and transcellular transendothelial migration

Paracellular transendothelial migration refers to the migration of leukocytes or cancer cell between adjacent endothelial cells. Transmigrating cells transmigrate between the tight junctions and adhering junctions. This process requires communication between migrating and endothelial cells. Many adhesion molecules and signaling receptors are shown to be involved in leukocyte paracellular transmigration, for instance the involvement of VCAM-1 (CD105) and ICAM-1 (CD54) signaling (Yonekawa and Harlan 2005), deletion of tight junction proteins such as VE-cadherin and activation of MLCK through increased cytosolic free calcium which creates a tension in the endothelial cells (Muller 2013). It was also reported that the aggregation of ICAM-1 triggered the activation of RhoA which in turn stimulated PP1phosphorelation by signals generated by ICAM-1. All these events lead to the initiation of contraction against the cell-cell junctions in between endothelial cells (Yonekawa and Harlan 2005; Muller 2013). This contraction creates a space for leukocytes to cross the circulation. CD31 is also proposed to play an important role in leukocyte transmigration and blocking of CD31 has been shown to significantly reduce the transendothelial migration ability of NK cells and monocytes (Muller 1995) but not lymphocytes (Bird et al., 1993). Extravasation is an important step in cancer cell metastasis where circulating cancer cells leave the circulation at the site of metastasis. The molecular
bases underlining this process are still not fully understood, but it is suggested that some cancer cells may mimic leukocytes during transendothelial migration (Tremblay et al., 2006). It was also reported that transmigration of cancer cells may cause irreversible damage to the intact barrier of endothelial cells due to the large size of the cancer cells compared to the size of leukocytes (Strell and Entschladen, 2008). Metastatic breast cancer cells are able to undergo paracellular transmigration which is accompanied by disruption of the endothelial barrier (De Bruyn and Cho, 1982).

Transcellular transendothelial migration refers to transmigration of the migrating cell through the endothelial cells. This occurs less frequently than paracellular transmigration. It is proposed that leukocytes may use this route as well (Ley 2007 et al.; Petri and Bixel 2006). ICAM-1 plays a key role in transendothelial migration by binding to the lymphocyte fusion-associated antigen-1 (LFA-1) expressed on the surface of leukocytes. This binding leads to relocation of ICAM-1 molecules which are more condensed at vesiculo-vacuolar organelles to establish the intracellular channels (Ley et al., 2007). It has been reported that leukocytes and cancer cells are both able to transmigrate through the endothelial cells (Khuon et al., 2010) as well as cross through the endothelial cells in transcellular migration without inducing endothelial cell death (Khuon et al., 2010). It is suggested that in transcellular migration both cancer cells and leukocytes use the same mechanism, which is dependent on endothelial cell cytoskeleton activity in response to adhesion of the migrating cell (Arvanitis et al., 2014).
1.9 Non-small cell lung cancer (NSCLC)

Lung cancer represents the most common cause of cancer-related deaths (Jemal et al., 2009). Histologically, lung cancer is classified into two major types: small cell lung cancer (SCLC) which represents 10% of lung cancer cases and Non-small cell lung cancer (NSCLCs) which is the most common type and represents 85% of lung cancer cases (Jemal et al., 2009). Approximately 70% of patients with NSCLC show multi-focal or metastatic tumours at the time of diagnosis (Molina et al., 2008). NSCLC is classified pathologically and genetically into distinctive three subtypes (Horn et al., 2012): adenocarcinoma, which originates from cells that line the peripheral tissue of the lung and secrete mucus. Most carcinoma cells appear with a glandular cell morphology with distinctive duct formation (Travis et al., 2004), this type of cancer represents about 40% of lung cancer cases and affects both smokers and non-smokers (Liu et al., 2010). The second subtype is squamous cell carcinoma, which develops from squamous epithelial cells of the lung. It is the most common type of NSCLC that affects smokers (Travis et al., 2004). The third subtype is the large-cell carcinoma which also arises from epithelial cells in the lung; cells appear with overabundant cytoplasm, large nuclei and prominent nucleoli (Travis et al., 2004). Roughly 9% of lung cancer cases are large-cell carcinoma. NSCLC is clinically subgrouped based on the development and spread of primary tumour (Tsim et al., 2010) to different stages. This classification is used to select the most appropriate treatment through providing essential information about tumour prognosis and patient response in clinical trials. The TNM staging system (T: tumour, N: lymph node and M: metastasis) was established by the international association for the study of lung cancer (IASLC) (Goldstraw et al., 2007 and AJCC).
This system depends on the morphological characteristics of the primary tumour (T), the involvement of lymph node (N) and the formation of metastatic secondary tumours (M) (Green et al., 2002; Sobin and Wittekind 2002). According to the most recent edition of the TNM system (7th edition), integration of three descriptors T, N and M are utilised to generally describe the stage of the disease as in table 1 (Goldstraw and Crowley, 2006) and table 2 (Goldstraw et al., 2007 and Tsim et al., 2010). The management of NSCLC patients varies depending on the stage of the disease, tumour prognosis and patient age. Surgery has a key role in patients who are diagnosed with early-stage (I-II) and (IIIA) NSCLC particularly (T3N1) (Scott et al., 2007) and pulmonary lobectomy is recommended for the patients at stage I-II and T1N0 NSCLC with no lymph node involvement (Scott et al., 2007). In some cases, radiotherapy is the most widely used choice in early-stage NSCLC (Rowell and Williams, 2001). Adjuvant chemotherapy was recently highlighted to increase the survival rate (5.4%) of early stage NSCLC particularly patients with stage II and IIIA disease. It has also been reported that adjuvant therapy is helpful to control the disease in patients with NSCLC at stage IB (Strauss et al., 2008). However, adjuvant therapy was seen to be correlated with poor prognosis in patients with NSCLC stage IA (Pignon et al., 2008). Chemoradiotherapy is applied as the standard therapy for patients with locally advanced NSCLC, such as stages IIIA and IIB. This regimen shows a high survival rate in patients with good physical fitness (Furuse et al., 1999). In patients at stage N2, surgery was seen to cause a high level of toxicity; in this approach, the European trial recommended three cycles of cisplatin-based chemotherapy followed by surgical resection or successive thoracic radiotherapy for the patients who were not fit enough for surgery (Furuse et al., 1999). Chemotherapy is the choice of treatment in metastatic NSCLC patients and has been reported to increase the survival rate to 9% at 12 months (Lilenbaum et al., 2005). A Cisplatin-based double regimen has also been correlated with one-year survival rates compared with regimens without platinum (which is usually used alternatively for patients who are sensitive to platinum) (Azzoli et al., 2009).
There is no unified standard regimen for patients with advanced NSCLC however, cisplatin/gemcitabine or cisplatin/pemetrexed regimens are recommended based on the histological characteristics of the tumour (Scagliotti et al., 2008). The regimen for patients with NSCLC brain metastases is chosen depending on the history of treatment that was previously used against primary lung tumour. Cisplatin is recommended for naïve patients (Kleisbauer et al., 1990) and Temozolomide (TMZ) is suggested to treat the recurrent NSCLC-brain metastases, with a 20% positive response (Giorgio et al., 2005). In addition, inhibition of the epidermal growth factor receptor (EGFR) was recently reported to elicit 27% response rate (Shimato et al., 2006).
<table>
<thead>
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<th>T Primary tumour</th>
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<tr>
<td><strong>T1</strong></td>
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<tr>
<td><strong>T1a</strong></td>
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<td><strong>T1b</strong></td>
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<td><strong>T2</strong></td>
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<tr>
<td><strong>T2a</strong></td>
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<td><strong>T2b</strong></td>
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<td><strong>T3</strong></td>
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**Regional lymph nodes (N)**

| N0 | No regional lymph node metastasis. |
| N1 | Involvement of ipsilateral hilar or peri-bronchial nodes. |
| N2 | Involvement of ipsilateral mediastinal or subcarinal nodes. |
| N3 | Involvement of contralateral mediastinal or hilar nodes or involvement of ipsilateral/contralateral scalene or supraclavicular nodes. |

**Distant Metastasis (M)**

| M0 | No distant metastasis. |
| M1 | Distant metastasis present. |
| M1a | Separate tumour nodule(s) in a contralateral lobe or tumour with pleural nodules or malignant pleural/pericardial effusion. |
| M1b | Distant metastases. |

Table 1: The TNM staging system of NSCLC showing the bases of staging protocol of NSCLC by patients grouping the depending on the features of primary tumour (T), lymphnode involvement and formation of secondary tumours (M) (Goldstraw et al., 2007 and Tsim et al., 2010).
<table>
<thead>
<tr>
<th>Stage</th>
<th>cTNM Subset</th>
<th>Five-year Survival</th>
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<tr>
<td>0</td>
<td>Carcinoma <em>in situ</em></td>
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</tr>
<tr>
<td>IA</td>
<td>T1a/T1b, N0M0</td>
<td>50-80%</td>
</tr>
<tr>
<td>IB</td>
<td>T2aN0M0</td>
<td>47%</td>
</tr>
<tr>
<td>IIA</td>
<td>T1a/T1b, N1M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2aN1M0</td>
<td></td>
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<td></td>
<td>T2bN0M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2bN1M0</td>
<td>36%</td>
</tr>
<tr>
<td>IIB</td>
<td>T2bN1M0</td>
<td>26%</td>
</tr>
<tr>
<td></td>
<td>T3N0M0</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>T1/T2,N2M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3,N1/N2,M0</td>
<td></td>
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<tr>
<td></td>
<td>T4,N0/N1,M0</td>
<td>19%</td>
</tr>
<tr>
<td>IIIB</td>
<td>T4N2M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Any T, N3, M0</td>
<td>7%</td>
</tr>
<tr>
<td>IV</td>
<td>Any T, Any N, M1a/M1b</td>
<td></td>
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</table>

Table 2: The clinical TNM subsets staging of NSCLC which goes in parallel with the subgrouping of cTNM subsets and the percentage of rate five-year survival (Goldstraw *et al.*, 2007 and Tsim *et al.*, 2010).
1.10 Brain metastasis

Metastasis is the main cause of death among cancer patients and correlates with poor prognosis (Liotta et al., 1993 and Hunter et al., 2008). Brain metastasis accounts for 25% of all metastatic cancers and secondary brain tumours are diagnosed in 20-40% of cancer patients (Salhia et al., 2014). Around 40%-50% of brain secondary tumours originate from lung cancer, 15%-20% from breast cancer, 5%-10% from skin cancer and 4%-6% from gastrointestinal tract cancer (Barnholtz-Sloan et al., 2004). In general, 80% of brain metastases are localised in the cerebral hemispheres, 15% in the cerebellum and 5% in the brainstem. It is suggested that the tissue volume of the brain or the amount of blood supplied to these parts may reflect these differences (Delattre et al., 1988). Secondary brain tumours are also frequently seen to present at grey-white junctions (Chang et al., 2007), where circulating cancer cells arrest or are trapped in small blood capillaries (Svokos et al., 2014). In patients with metastatic non-small-cell lung cancer (NSCLC), synchronous brain metastases are diagnosed in 35% and metachronous brain metastases in 65% of cases (Burt et al., 1992). The survival rate varies among patients: 55% of NSCLC patients with brain metastasis survive for 1 year; 27% for 2 years; 18% for 3 years and 13% for 5 years (Burt et al., 1992 and Ali et al., 2013). Circulating cancer cells enter the brain through haematogenous pathways via the blood brain barrier capillaries (Wilhelm et al., 2013). However, in mice malignant lymphoma cells have been seen to enter the brain through the choroid plexus and cranial nerve (Hochman et al., 2001) and recent reports are highlight the possibility of existence of lymphatic vessels network in mouse brain (Aspelund, et al., 2015).
Three pioneering theories were proposed to explain the mechanism of metastasis.

(1) The “seed-and-soil” theory proposed by Stephen Paget (1889) hypothesised that, metastasis does not occur randomly, but rather cancer cells target specifically certain organs to initiate secondary tumours. This theory considers the cancer cell to be the “seed” and the site of secondary tumour to be the “soil”. It is based on studying 735 cases of advanced breast cancer (Stephen Paget, 1889 reviewed by Mendoza and Khanna, 2009); the “seed-and-soil” theory also explains the selectivity of initiating secondary tumours in specific organs but does not explain why not all cancer cells are able to form secondary tumours. In fact, less than 0.01% of circulating cancer cells are able to initiate new metastases (Chambers et al, 2002). In a quantitative study, one gram of rat mammary adenocarcinoma primary tumour may release approximately $4 \times 10^6$ metastatic, circulating tumour cells (Butler and Gullino, 1975).

(2) The “mechanical entrapment” theory proposed by James Ewing (1923) speculates that metastasis is purely a mechanical process and cancer cell dissemination depends on the circulation, it also explains why not all circulating cancer cells are able to initiate a new secondary tumour. However, it does not explain why cancer cells seed some organs rather than others.

(3) The third theory proposed by Duda (2010) states that “the metastatic cancer cell “seeds” grow preferentially in secondary sites within a “permissive microenvironment”’. He also reported that metastatic cancer cells could bring their own soil from primary site such as activated fibroblasts (Duda et al, 2010).
1.10.1 Diagnosis of brain metastases

Three main types of examinations are applied to diagnose a brain tumour; they include a neurological examination, brain scan, biopsy and pathology examination.

- **A neurological examination**: it includes a physical examination, medical history as well as, a group of different tests used to assess the function of the nervous system, sensory and motor responses particularly reflexes. In case of any abnormalities, the patient will be referred for further investigations such as brain scan (Nicholl and Appleton 2015).

- **A brain scan (neuroimaging)**: it refers to imaging internal structures of the brain. Brain scan is conducted by using highly specialised and computed devices. There are many types of scans are established since Hounsfield and Ambrose launched the axial tomography (CAT or CT scanning) technique in 1971. CT scan is still used for a quick and general scan to investigate the brain structure (Filler 2009). Diffuses optical imaging or tomography (DOI) or (DOD) is another brain scanning technique, that is employ the near infrared light in imaging and depends on the optical absorbance of haemoglobin based on various oxygenation level. Magnetic resonance imaging (MRI) also is used in brain scanning, it based on using radio waves and magnetic fields to generate two- or three-dimensional images. Some of brain scans employ a contrast agents or dyes, which are useful to differentiate the abnormal brain tissue, for example the gadolinium-based medium is the most common used in MRI brain scan (Mitsumori et al., 2014).

- **Biopsy and pathology examination**: A biopsy is a surgical operation, which is conducted to obtain a tissue sample from the site of the tumour. In biopsy test, the tissue is usually fixed, stained and microscopically examined. The pathological analysis of the biopsy provides information on the type and the grade of the tumour (Filler 2009).
1.10.2 Treatment approaches of brain metastases

The treatment options of brain metastasis are limited and tend to be local therapy (Berghoff AS and Preusser, 2015). Management of brain metastasis may include one or more of the following treatments:

1.10.2.1 Surgery

Once a metastatic brain tumour has been diagnosed, the patient may undergo surgery depending on the tumour size and location (Schuette 2004). In general, surgical removal of secondary brain tumour is used to manage an accessible, single or two, large, life threatening brain tumour; surgical resection aims to eliminate the neurological symptoms caused by the tumour. In addition, the candidate has at least six-month life expectancy and well enough to tolerate the surgery (Caffo et al., 2013). Surgery significantly reduces the neurological symptoms, renovates the flow of CSF, mitigates symptomatic intracranial hypertension and lessen peritumoral edema and steroid dependence. Furthermore, surgery offers is helpful in conforming the correct pathological diagnosis. However, surgical resection is a limited choice in the case of multiple brain metastases (Adenot et al., 2007).

1.10.2.2 Radiotherapy

Radiotherapy is the most commonly used treatment in brain metastasis and the whole brain radiation therapy (WBRT) is routinely used in treatment of patients with brain metastases, It aims to treat the diagnosed secondary brain tumours as well as to prevent the new ones (Berghoff AS and Preusser, 2015). Treatment with only WBRT is seen to increase survival rate up to six months (Caffo et al., 2013) although, 11% of the patients develop late brain toxicity following the WBRT. Recently, it was reported that 23% of patients with brain metastases at a risk to develop a radiation-refractory and post-radiation leukoencephalopathy, which are defined by gait disturbance, dementia, incontinence in addition to, attention and executive deficit (Spiceland et al., 2015). However, that does not reduce the importance of radiotherapy in fact; WBRT following a surgical resection is
considered as an effective treatment for a single accessible metastatic brain tumour for patients with controlled primary tumour and well condition (Gaspar et al., 2010). Moreover, WBRT is seen to control the presenting neurological symptoms in 70-90% of patients with multiple brain metastases without causing acute neurological symptoms side effects (Hoegler, 1997). Treatment with prophylactic cranial irradiation (PCI) reduces the incidence rate of developing brain metastasis in patients with small cell lung cancer (SCLC) from 18% to 8%; unfortunately, PCI deteriorates memory function in some patients (Sun et al., 2011). There are different WBRT protocols which are prescribed based on the patient general condition and the nature of the secondary tumour; a short radiation therapy courses with 20 Gy to 30 Gy total dose which is usually given in a 300 to 400 cGy fractionated dose (Hoegler, 1997).

1.10.2.3 Radiosurgery

It is a radiotherapy treatment technique, in which, a high dose of ionised radiation selectively applied on a targeted tissue (tumour). Stereotactic radiosurgery (SRS) is considered as a successful short-term treatment option. Unlike WBRT, the SRS targets a specific tissue area with less than 3.5 cm diameter, using a multiple cobalt-60 sources (gamma-knife) or a linear accelerator (Caffo et al., 2013). SRS is highly recommended choice for patients with inaccessible brain tumours or, for patients who unable to tolerate surgery or patients with resistant metastatic brain tumours. SRS showed a high control rate of local tumours, up to a 85% control rate at 12 months and a 65% control rate at 24 months (Alexander et al., 1995). Cyberknife (CK) is a recent radiosurgery treatment technique, in which higher radiation doses applied directly to the tumour. Treatment with single-fractionated doses about 10-36 Gy showed a high control rate up to 95% in patients with metastatic brain tumours (Chang et al., 1998). Another study showed a symptomatic improvement occurred in 90% of patients with a 77.8% control rate following treatment with CK (Wang et al., 2009).
1.10.2.4 Chemotherapy

Chemotherapy is known for its limited potency in treatment of cerebral metastasis due to the effect of blood brain barrier, drugs delivery is seen to be interrupted or turned over quickly in the brain or not distributed equally. Chemotherapy is often applied for patients with poor response to other treatments. However, in some cases systematic chemotherapy has shown a response rate of 50% in patients with cerebral metastasis from NSCLC and SCLC (Schuette 2004). Chemotherapy efficacy varies according to the sensitivity of secondary tumour cells to the drug of choice; brain metastases that originated from lung (NSCLC) and breast are tend to be less sensitive to chemotherapy (Caffo et al., 2013). A previous study reported that cisplatin and etoposide gave a significant objective response rate at 55% in breast to brain metastases (Boogerd et al., 1992). Topotecan is a new chemotherapy, it has the ability to cross the BBB and used to control primary and secondary brain tumours; it is a semi-synthetic camptothecin derivative. Topotecan inhibits topoisomerase I during S-phase of cell cycle causing cell proptosis (Ardizzoni et al., 1997). However, topotecan is effective in treatment of brain metastases originated from SCLC and less effective in metastases from other organs (Wong et al., 2004). Temozolomide (TMZ) is an alkylating agent with a small molecular weight, which enables it to cross the BBB (Lorusso et al., 2006). TMZ is associated with low incidence of side effects and it has been suggested that TMZ may decrease brain metastases (110). A recent study showed that afatinib can be used in treatment of brain metastatic NSCLC with EGFR mutations (LUX-Lung 3) (Schuler et al., 2016).
1.10.2.5 Molecular Targeted Therapy

Overexpression of many receptors and activity of some signalling pathways have been demonstrated, to be associated with brain metastasis such as, tyrosine kinase receptors, which trigger many oncogenic pathways. A specific blocking of these receptors and pathways could interrupt proliferation, invasion, and migration of cancer cell. These new target therapies inhibit the activation of oncogenic pathways through; ligand-receptor binding or by blocking downstream signal transduction in order to prevent the disease progression. In theory, the molecular targeted therapies are designed to be highly specific and safer with less systemic cytotoxic effect (Caffo et al., 2013).

- **Trastuzumab** is human monoclonal antibodies against extracellular domain of human epidermal growth factor receptor 2 (HER2), this receptor overexpression is noted to be correlated with formation of brain metesteses in breast cancer patients (Caffo et al., 2013). Recently, trastuzumab is used either alone or combined with chemotherapy in treatment regimen of breast cancer patients. A retrospective study showed that trastuzumab is significantly effective in HER2-positive breast cancer patients but not in HER2-negative patients (Caffo et al., 2013).

- **Lapatinib** is another molecular targeted therapy; it is a small-molecule tyrosine kinase inhibitor that blocks the phosphorylation of both, epithelial growth factor receptor EGFR and HER2, as well as prevents the downstream-signalling proteins that trigger cell proliferation and migration (Schuler et al., 2016). Lapatinib has the ability to cross BBB (Caffo et al., 2013) and it is used when patients develop trastuzumab resistance (Caffo et al., 2013). Lapatinib is recently used to treat HER2-positive breast cancer patients with brain metastasis, at dosage of 750 mg twice daily after a cranial radiation (Caffo et al., 2013).
- **Bevacizumab** is a monoclonal antibody that is designed to bind vascular endothelial growth factor receptor b (VEGF-b), in order to prevent formation of new blood vessels during angiogenesis process (Caffo *et al.*, 2013). Bevacizumab is associated with a risk of developing an intracranial hemorrhage, as it has been reported in a phase II trial of bevacizumab in which, the use of this treatment was excluded in NSCLC patients with brain metastasis (Caffo *et al.*, 2013).

- **Multi-target Tyrosine Kinase Inhibitors** are mainly target Mitogen-activated protein kinases (MAPK) pathway which is a key intracellular signal transduction pathway; it is involved in cell proliferation, differentiation, mitosis, gene expression and apoptosis (Zahuo *et al.*, 215). Sorafenib or sunitinib are active inhibitors of multi-tyrosine kinase inhibitors, they inhibit cell proliferation via blocking the activity of intracellular kinases in Raf/MEK/ERK pathway (134); it is also reported that Sorafenib or sunitinib prevent angiogenesis by inhibiting VEGFR-2, VEGFR-3, FLT, FMA, RET, c-KIT and PDGFR-b. A CNS hemorrhage was reported in metastatic renal cell carcinoma (RCC) patients who, were treated with Sorafenib or sunitinib (Zahuo *et al.*, 215). LGX818, dabrafenib and vemurafenib are potent inhibitors of mutated BRAFV600E but not BRAF-wild type cells, unfortunately these inhibitors are associated with a specific toxicities and drug resistance (Zahuo *et al.*, 215).

- **Rapamycin and its analogs** (mTOR inhibitor) are anti-tumour, lipophilic molecules, they can cross the BBB. mTOR inhibitors (at high concentration) reduce the invasion of metastatic breast cancer cells *in vitro* (Caffo *et al.*, 2013). *In vitro*, a high dose of mTOR inhibitors CCI-779 combined with MEK inhibitor (SL327) reduced perivascular invasion and angiogenesis (Zahuo *et al.*, 215).
1.11 Metastasis mechanism and pathways

Metastasis is a complex multi-step process; inducing the development of secondary tumours from a distant primary tumour (Stephen Paget, 1889 reviewed by Mendoza and Khanna, 2009). Metastasis is the main cause of death among cancer patients and correlates with poor prognosis and treatment outcomes (Liotta et al., 1993 and Hunter et al., 2008). The molecular mechanisms underlining metastasis are still ambiguous, although numerous studies have highlighted that the cancer cells disseminate through two modes: (a) the “serial model” (Liotta et al., 1993 and Pavelic et al., 2011) in which, the metastatic cells arise from a primary tumour and metastasis is the terminal stage of tumuorigenesis; this model does not explain the genetic differences between primary tumour and the new secondary tumour (Schmidt-Kittler et al., 2003 and Gray 2003). (b) “Parallel” model, where the dissemination of cancer cells occurs in parallel with primary tumour progression. This model may explain the early formation of secondary tumours but does not explain why metastasis occurs at an advanced stage of primary cancers.

Once metastatic cancer cells successfully detach from the primary tumour, they may use one or more pathways to disseminate and reach the site of the secondary tumour. These pathways or routes can be summarised as: tissue spaces (Transcoelomic) which refers to the infiltration of metastatic cancer cells by invading the compact tissue which may also include the fibrous membrane of the periosteum as in bone metastases (Liu et al., 2015), pericardium as in cardiac metastases (Bussani et al., 2007), dura matter (Agarwal et al., 2010), ligaments (Akhavan 2013), tendons (Jozsa and Renner 1991), derma (Sesterhenn et al., 2013) and viscera that surrounds the liver, kidneys and spleen (Drake 2015). Cancer cells potentially destroy these barriers via proteolytic enzymes (Morgan-Parks, 1995).

An additional potential route for brain metastasis is via the cerebrospinal spaces by metastatic cancer cells through cerebrospinal fluid (CSF) by invading the leptomeninges.
surrounding the brain, spinal cord and ependymal lining of the brain ventricles (Pilkington and Parker 2007).

Epithelial cavities constitute another pathway of cancer cell dissemination which occurs by direct implantation or contact of cancer cells on the epithelial surface, this type is rarely seen in the digestive and respiratory system due to the heavy mucus secretions and bacterial normal flora (Morgan-Parkes 1995). Lymph vessels (lymphatic spread) another pathway of cancer cell dissemination. In this path cancer cells do not require any proteolysis activity to enter the lymphatic drainage, since lymphatics do not have a continuous basement membrane (Oliver and Harvey 2002). There is in depth knowledge that metastasis to the lymph nodes occurs before or in parallel with the diagnosis of malignant primary tumours. Therefore, it is considered as an important diagnostic indicator of metastasis in some types of cancer (Fidler 2003 and Shayan et al., 2006). Some cancer cells express specific receptor molecules such as CCR7 which increases the affinity towards lymphatic vessels as in breast cancer and melanoma (Wiley et al., 2001). From cancerous lymphatic nodes, cancer cells frequently invade the blood circulation, through the blood venules, lymphaticovenous anastomoses in the lymph node, neighbouring veins and lymphatic tributaries of veins (Morgan-Parkes 1995). A haematogenous route in which the spread of circulating cancer cells occur through blood circulation starts with angiogenesis where the primary tumours are in direct contact with the blood vessels due to the angiogenesis process. Circulating cancer cells are detected in blood samples in 50% of cancer patients at the time of primary tumour diagnosis (DeVita et al., 1975). Cancer cell transmigration does not occur via arteries, since the elastic fibrous area surrounding large blood vessels contain anti-proteolytic factors (Willis, 1973 and Morgan-Parkes, 1995). Venous invasion occurs in most cancer types. Cancer cells invade the small venules surrounding primary tumours either in single cell form (Hart 2009) or in clumps to form a tumour embolus, which subsequently arrests in the nearest venous (Hart 2009). The site of primary tumour also plays an important role in spread of cancer cells, for instance, cancer
emboli that are released in systemic veins are seen to arrest in the lungs (Heithaus et al., 2013), while those released in the portal venous system are generally arrest in the liver (Morgan-Parkes 1995). Cancer emboli released in pulmonary veins tend to arrest in various sites in peripheral tissues such as the brain or even the bone (DAntonio et al., 2014).

1.11.1 Initial steps of metastasis

Metastasis is a complex, multi-step process where a metastatic cancer cell has to undergo various steps of cancer cell-host interactions (Liotta et al., 1989). Interrupting any of these steps may prevent the formation of secondary tumours (Figure 8). Local invasion represents the first step to metastasis where malignant cells start invading the surrounding tissue aligned with the primary tumour. Cancer cells are able to digest the extracellular matrix and the basement membrane, the intestinal stroma and bone as invading cancer cells attach themselves to the basal membrane or extracellular matrix using tumour cell surface receptors (Morgan-Parks 1995). The locomotion of invading cancer cells may be influenced by self-derived motility factors (autocrine) as in human melanoma cells (Stetler-stevenson 1989 and Adada et al., 2015) or influenced by host-derived motility factors (paracrine) as in hepatocyte growth factor (HGF) which stimulates the migration of lung carcinoma, colon and melanoma cells to the liver (Ogasawara and Suzuki 2004). The growth of the primary tumour induces formation of new host-blood capillaries into the tumour mass in a process called angiogenesis, which involves host endothelial cell migration, growth and differentiation and is a crucial process in the progression and growth of a local tumour in becoming a large and potentially malignant tumour (Folkman 1994). It is revealed that formation of new vessels is critical for a tumour to grow larger than 2 mm diameter and inhibition of angiogenesis reduces tumour growth (Muthukkaruppan et al., 1983). Angiogenesis is induced by tumour and stromal cells via secretion of angiogenesis factors which are controlled by activators and inhibitors (Dameron et al., 1994). The
vascular endothelial growth factor (VEGF) family is considered to play a major role in angiogenesis in cancerous tissue (Folkman 1990) and a significant correlation is reported between cancer progression and upregulation of VEGF in many cancers such as lung (Decaussin et al., 1999), breast (Kurebayashi et al., 1999) and colorectal cancer (Andre 2000). Some cancer cells are able to escape from the local tumour and enter into the circulation via invading the local blood or lymphatic vessels through a process called intravasation (Reymond et al., 2013), it was reported that the number of secondary tumours is correlated with the number of progressive circulating cancer cells (Liotta et al., 1991) and newly formed blood vessels during angiogenesis are potentially the favorite target for metastatic cells since the new vessels are imperfectly formed with many abnormalities (Netland and Zetter 1989). It was also shown that intravasation can occur either actively in which only viable cancer cells invade the circulation and there is considerable evidence, showing the involvement of cytoskeletal activation (Condeelis and Pollard 2006) integrin upregulation (Hood and Cheresh 2002) during intravasation; or may occur passively where cancer cells both dead and viable enter into the circulation. The passive intravasation has been reported to occur when parts of a tumour mass impacts upon a tenuous blood vessel and that may explain why only 1% of circulating cancer cells are viable cells (Bockhorn et al., 2004) (Figure 8).
Figure 8: Initial steps of metastasis steps at the site of the primary cancer

Figure 8: Schematic shows metastasis steps at the site of primary tumour. (1) Local invasion starts at primary tumour site by degradation of basement membrane and ECM. (2) Uncontrolled proliferation of malignant cells takes place followed by angiogenesis. (3) Intravasation where some of cancer cells succeed to enter the circulation.
1.11.2 Metastasis steps within the circulation and at entry site of secondary tumour

Millions of malignant cells are distributed from the site of the primary tumour, either in single cell form (Morgan-Parks 1994) or in clumps (Aceto et al., 2014). The blood represents a hostile environment for circulating tumour cells (CTCs) (Fidler 2003 and Mehlen and Puisieux 2006) and less than 0.01% of total CTCs form a secondary tumour (Fidler 2003 and Liotta 1989). In circulation, platelets and leukocytes play an important role in survival of CTCs, for instance cancer cells expresses VCAM-1 which binds to α4 integrin on the surface of macrophages and this interaction protects the cancer cell against the effect of the pro-apoptotic cytokine TRAIL (Chen et al., 2011). Cancer cells stop traveling in the circulatory system after a while through a process called cancer cell arrest. Two major patterns of cancer cell arrest are described: passive CTC arrest, where the disseminated cancer cells arrest at the nearest capillary beds, which are a network of fine capillaries where the gaseous and nutrients exchange occurs. It is also characterised by slow rate of blood flow. These beds may represent an ideal adhesion site for the circulating cells in blood capillary of the primary tumour. This occurs mechanically and is caused by trapping individual cells or groups of clumping cancer cell in capillaries with small-diameter as in metastasis from breast to lung and from colon to liver (Chambers et al., 2002). It has however, also been reported that lung cancer cells may arrest in the large-diameter vessels (Al-Mehdi et al., 2000). The other pattern is active arrest of cancer cells where CTCs actively adhere to the vascular endothelium, via specific cell-cell adhesion molecules such as the interaction of selectins with their ligands: Sialyl Le x and Sialyl Le a (Orr and Wang 2001), integrins (Felding-Habermann et al., 2001). Active cancer cell arrest is detected in many cancers and may explain the selectivity of cancer cell adhesion; for example CD62E, ICAM-1, VCAM-1, ALCAM, β4 integrin and VLA-4 which are shown to play a key role in the adhesion process of breast cancer cells to brain endothelium during metastasis from breast to brain (Soto et al., 2014). CD15 and CD62E are also localised at
the site of adhesion between brain endothelium and NSCLC cells during metastasis from lung to the brain (Jassam et al., 2015).

Furthermore, arrest and adhesion of cancer cells are seen to be correlated with the presence of specific cytokines such as TNF-α, IL-1α and IL-β that induce the expression of E-selectin, P-selectin, ICAM-1 and VCAM-1 at the surface of endothelial cells (Iwai et al., 1993). Approximately, 90% of CTC arrest at capillaries or at the wall of a vessel (Morgan-Parkes, 1995). It is reported that arrested CTCs start degradation and invasion of the lamina (basal membrane) of vessels after 8-24 hours of adhesion on the endothelial cells in a process called extravasation (Liotta, 1989); extravasation is controlled by the type of cancer cell and the host-inflammatory reactions such as secretion of TNF-α which is known to regulate adhesion of cancer cells (Bissell et al., 2005). A varying degree of ability of extravasation is seen in different cancer cells, for instance lung metastatic cancer cells are able to disrupt the integrity of endothelial barrier by expressing VEGF-A and Angpt14 factors which are known to affect the junction between endothelial cells during cancer cell endothelial transmigration (Weis et al., 2004 and Padua et al., 2008) and EREG, COX2, MMP1 and MMP2 which have been reported to mediate the extravasation of lung cancer cells (Gupta et al., 2007). It is proposed that direct contact of cancer cell-platelets activates both NF-κB and TGFβ/Smad in cancer cells which triggers the epithelial-mesenchymal transition in lung cancer cells in vitro. Cancer cells may thus acquire a “invasive mesenchymal phenotype” in vivo. The absence of TGFβ1 also significantly reduced the extravasation of lung cancer cells (Labelle et al., 2011). In colon carcinoma metastasis, cancer cells express CCL2 (monocyte chemotactic protein 1) which directly affects CCR2 that is released by endothelial cells; both proteins are seen to be correlated with increased permeability of the endothelial cell barrier (Wolf et al., 2012) (Figure 9).
Figure 9: Metastasis steps during the circulation and at site of entry of secondary tumours

1. Local Invasion
2. Proliferation & Angiogenesis
3. Invasation
4. Survival & Education
5. Tethering & Rolling
6. Extravasation
7. Cell growth

Figure 9: Metastasis steps at circulation and entry to the site of secondary tumour. (4) Survival and education of cancer cells in the circulation through interacting with leukocytes and platelets. (5) Tethering and rolling of cancer cells followed by adhesion on the endothelium of the capillary. (6) Extravasation, some cancer cells are able to leave the circulation by transendothelial migration. (7) Cell growth of metastatic cancer cells at the site of secondary tumour.
1.11.3 Seeding of cancer cells into the brain

Little is known regarding the mechanisms underlining the entry of cancer cells into the brain during the early stage of metastasis to the brain. Recent studies have, however, highlighted the role of endothelial cells. It was shown that activated brain microvascular endothelial cells expressing CD62E plays an important role in extravasation as they mediate cell-cell adhesion between cancer-endothelial cells (Brodt et al., 1997 and Jassam et al., 2015). Vascular adhesion molecules such as ALCAM, ICAM-1, VLA-4, VCAM-1 and β4 integrin, have been shown to play an important role in mediating cancer cell extravasation, via initiation of adhesion of breast cancer cells to brain endothelium (Soto et al., 2014). Although most of the cells of the brain develop from common neuroepithelial cells of the embryonic nervous system, the brain still has a distinctive cellular asymmetry (Gotz and Huttner, 2005). According to the differences in morphology and functional characteristics, brain cells are categorised into two major types: Neurons: characterised large cell body, prominent transmitting axon and processes dendrites (Ferris, 2012); there are at least 100 types of neurons (Kandel et al., 2012) and glial cells which can be classified into: microglia which constitute some of the phagocytes of the brain (Banati, 2003) and macroglia which include: astrocytes, oligodendrocytes and ependymal cells (Noble et al., 1990). Glial cells function as supportive cells. The brain cells receive solutes and oxygen through a unique circulation system incorporating the neuro-vascular unit which constitutes the blood brain barrier (Abbott et al., 2002).
1.12 Structure and function of the blood brain barrier (BBB)

The blood brain barrier (BBB) represents the first line of defence in the central nervous system (CNS) and any disorder which leads to disruption of the BBB can cause many CNS dysfunctions (Wilhelm et al., 2013). The BBB helps regulate the homeostasis of the CNS as it controls the entry of solutes and cells between the circulatory system and neuronal tissue (Lee et al., 2006). It is composed of the neurovascular unit (Abbot et al., 2012), which consists of three fundamental elements: endothelial cells, pericytes, and astrocytes (Figure 10). The transportation process across the BBB is strictly controlled by several barriers including the interendothelial tight junctions (TJs) between endothelial cells. TJs are composed of four transmembrane proteins (claudins, occludin, tricellulin/marvelD2 and marvelD3) which regulate the entry of water soluble molecules (Willelm et al., 2011).

In addition, transcellular barrier, another defence line, is characterised by a low level of transcytosis and endocytosis within cerebral endothelial cells which inhibits the transport of substances via the cytoplasm. The enzymatic barrier consists of a complex group of enzymes such as, alkaline phosphatase, gamma-glutamyl transpeptidase, acetylcholinesterase, monoamine oxidases and other metabolizing enzymes which are able to catalysis variety of drugs and chemicals. Efflux transporters are also considered as a defence line, expressed by cerebral endothelial cells and include: ABC-B1,-C1,-C4,-C5 and G2 and regulate the gaseous exchange, transport of nutrients and elimination of metabolites. The transport of amino acids and polar molecules such as glucose, are regulated actively by solute carrier proteins (SLC transporter family) (Wilhelm et al., 2011).
Figure 10: Schematic of the cellular structure of the BBB and cells involved in its function and integrity. Endothelial cells are in contact with pericytes on the basal lamina and the end-foot of astrocytes to form the morphological basis of the BBB, interneurons, microglia and perivascular macrophages are also seen to be distributed around the neurovascular unit.
The BBB has a unique cellular structure, starting with highly specialised cerebral endothelial cells (CECs) that line the lumen of BBB vessels. Unlike other non-cerebral endothelial cells, CECs have a high number of mitochondria (Oldendorf 1977), a reduced number of caveolae (Nag 2003), low level of pinocytosis (Nag 2003) and are tightly connected by the tight junctions (TJs), which are localized at the apical side of the BBB capillaries (Brightman and Reese 1969). In terms of BBB permeability, CECs are considered the most important cell type, as they form a continuous sheet lining the inner surface of BBB capillaries. The permeability of the brain endothelial monolayer can be measured by the value of transendothelial electric resistance (TEER). CECs also possess endothelial-like characteristics similar to the other non-CNS endothelial cells; such as expressing factor VIII (anti-hemophilic factor AHF), von Willebrand factor, high activity of alkaline phosphatase, high activity of γ-glutamyl transpeptidase and uptake of lipoproteins (Wilhelm et al., 2013). The second cell type of the BBB is the pericyte which is contractile cell related to smooth muscle. The role of pericytes in the formation and function of BBB is still not fully explored; however these cells are located at the basement membrane of BBB capillaries in close contact with the endothelium. Gap junctions between both cell types have been reported (Cuevas et al., 1984) and in BBB the ratio between pericytes to endothelial is about 1/3-5 (Pardridge 1999). It is reported that the absence of pericytes leads to endothelial hyperplasia, increased permeability of the BBB and abnormalities in vasculogenesis (Hellstrom et al., 2001 and Armulik et al., 2010). The interaction between pericytes and endothelial cells is reported to play an important role in regulating the BBB during embryogenesis (Daneman et al., 2010). Astrocytes also play an important role in the stability of BBB (Abbott et al., 2006 and Krizbai et al., 2012). In coronal brain sections in the rat, the end-feet of astrocytes are seen to fold around the BBB capillaries and cover both endothelial and pericyte cells (Kacem et al.,1998) as well as being in contact with nerve cells and with basal membrane (Cohen et al.,1998; Paspalas and Papadopoulos, 1996). High levels of the P-Kir 4.1 K⁺ channel glycoprotein (P-gp),
glucose transporter 1, aquaporin-4 and connexin-43 are detected on the adhesion site of astrocytic end-feet and endothelial cells. It was shown that the outer side of cerebral endothelial cells are folded within the basement membrane which is composed mainly of proteins such as collagen type IV, fibronectin, tenascin, proteoglycans and laminin (Nag, 2003). Basal membrane symbolizes anchor on which endothelial cells adhere via the interaction between endothelial integrins and the proteins of the basement membrane (Hynes, 1992). These interactions regulate many signaling events and expression of tight junction proteins described below (Tilling et al., 2002 and Savettieri et al., 2000). It is also reported that the basal membrane plays a vital role in regulating adhesion and migration of endothelial cells as well as in arresting and colonization of metastatic cancer cells during metastasis to the brain (Carbonell et al., 2009). The ECM proteins and receptors are expressed at the neurovascular unit play a pivotal role in regulating the growth and motility of the BBB cells as well as, they are involved in connecting the cellular and matrix compartments of the BBB (Baeten and Akassoglou 2011). ECM proteins and receptors are secreted by the component cells of the BBB, for instance, endothelial cells excrete fibronectin (Webersinke et al., 1992), collagen (Kose et al., 2007), laminin, laminin α4, laminin α5 (Tilling et al., 2002), agrin (Stone and Nikolics 1995), nidogen-1 (Stratman et al., 2009) and SPARC (Vincent et al., 2008). Endothelial cells also secrete the matrix receptors, dystroglycan (Engelhardt and Sorokin 2009) and integrins α1β1, α4β1, α5β1, α6β1, α6β4, αvβ1, αvβ3 (Paulus et al., 1993, Wang and Milner 2006, Milner and Campbell 2002, Tagaya et al., 2001). Additionally, ECM and matrix receptors are secreted by pericytes; fibronectin (Webersinke et al., 1992), glycosaminoglycans, collagen, laminin (Allt and Lawrenson 2001), nidogen (Brachvogel et al., 2007) and perlecan (Stratman et al., 2009). Pericytes have been shown to express matrix receptors such α4β1 and α4β2 integrins (Grazioli et al., 2006 and Balabanov et al., 1996). Astrocytic cells are shown to generate collagen (Kose et al., 2007), fibronectin (Webersinke et al., 1992), laminin (Tilling et al., 2002), agrin (Grimpe et al., 1999), SPARC (Vincent et al., 2008) and
nidogen-1 (Wolburg et al., 2009) as well as matrix receptors such as, dystroglycan (Engelhardt and Sorokin 2009) and integrins (Paulus et al., 1993).

Finally, microglia cells are reported to generate ECM and matrix receptors such as SPARC (Vincent et al., 1992), αvβ3, αvβ5, αvβ8, αmβ2, αLβ2 and αXβ2 (Zhu et al., 2009 and Milner et al., 2009). The BBB also has a unique junctional complex that is localised in the cerebral interendothelial space at the neurovascular unit, which is composed mainly of adherent junctions (AJs) (Schulze and Firth, 1993), tight junctions (TJs) (Kniesel and Wobblurg, 2000) and gap junctions (Tao-Cheng et al., 1987). TJs and AJs are both involved in regulating the permeability of the endothelial layer while, gap junctions are thought to play a role in intracellular communication (Bazzoni and Dejana, 2004). AJs facilitate the cell-cell adhesion of endothelial cells in the endothelial monolayer and inhibition of AJs leads to polarisation of endothelial cells (Bazzoni and Dejana, 2004). AJs are composed of cadherin which initiates cell-cell adhesion as it binds with other cadherin molecules on the adjacent endothelium; the intracellular part of cadherin binds to plakoglobin and β-catenin which is connected to the actin cytoskeleton of the endothelium (Wataba-Uchida et al., 1998). Disrupting the AJs increase the permeability of the BBB (Abbruscato and Davis 1999).

TJs are localised at the apical region of the endothelial barrier tissue. They play a key role in tightly binding the endothelial cell to facilitate a “zipper” like structure which isolates the apical region from the basolateral region of the endothelium. TJs consist of the following protiens: junctional adhesion molecule (JAM-1) which is a 40 kDa IgG and has a single membrane-span chain and a large extracellular domain. Its main function is to mediate the initial cell-cell adhesion via homophilic interactions. JAM-2 and JAM-3 related proteins are localised on the surface of epithelial cells but their role in the BBB is still not fully understood (Bauer et al., 2004). Occludin is a 60-65 kDa protein which has 4 transmembrane domains characterised with carboxyl and amino terminal ends and
extracellular loops (Furuse et al., 1993). Occludin is seen to be overexpressed and continuously distributed on the marginal edges of cerebral endothelial cells (Lippold et al., 2000) whereas a faint and discontinuous expression is detected in non-cerebral endothelium. It is revealed that overexpression of occludin is correlated with high electric resistance in endothelial tissues (Hirase et al., 1997). Claudins are a group of 20-24 kDa proteins which have similar structure to occludin without the homologous sequence (Furuse et al., 1998). At least 24 claudins are known in mammals, all of them sharing a similar predicted folding (Heiskala et al., 2001). The Claudin extracellular loop may initiate a homophilic or heterophilic interaction with different molecules. It is shown that claudins form the primary scaffold of TJs and occludin facilitates the additional seal of the TJs. Claudin-1,-3 and -5 are characteristic of the cerebral endothelium (Witt et al., 2003). TJs also contain membrane-associated guanylate kinase proteins (MAGUK) and three other proteins (ZO-1, ZO-2 and ZO-3). These proteins are recognised by a Src-homolog-3 domain, multiple postsynaptic density-95 binding domains and are shown to be associated with the structure of tight junctions such as ZO-1 (220 kDa) which is localised at the site of TJs (Stevenson et al., 1986).
PLAN OF INVESTIGATION
**Hypothesis**

1- CD62E plays an important role in cancer cell adhesion during metastasis from lung to brain.

2- The interaction between CD15/CD15s and CD62E may play important role in adhesion and trans-endothelial migration of lung cancer cells (Figure 11 A).

3- Interrupting the interaction between CD15/CD15s and CD62E may play an important role in adhesion and trans-endothelial migration of lung cancer cells through brain vascular endothelium (Figure 11 A, B).

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**Figure 11: Metastasis from lung to brain**

![Schematic representation of the metastatic cascade/progression of lung cancer cells metastasising to the brain. The diagram above shows key steps of metastasis process, focusing particularly on the role of CD15/CD15s and CD62E interaction in adhesion (A) and transendothelial migration (B) of circulating lung cancer cells.](https://example.com/schematic_diagram.png)
Aims

1- To characterise the extracellular expression of CD15, CD15s and CD62E both qualitatively and quantitatively in human non-neoplastic adult astrocytes (SC-1800), human brain microvascular endothelial cells (hCMEC/D3), human primary lung cancer cells (COR-L105 and A549), human GBM cells (UP-007 and SNB-19), human metastatic lung to lymph node (NCI-H1299) cells and metastatic biopsy-derived lung cancer cells to the brain (SEBTA-001 and SEBTA-005), tissue sections of normal brain and lung to brain secondary tumours.

2- To evaluate the effect of TNF-α on expression of CD62E in brain endothelial cells.

3- To investigate the effect of mAb-blocking of CD15/CD15s on adhesion of primary and metastatic lung cancer cells to brain endothelial cells.

4- To determine the effect(s) of mAb-blocking of CD15/CD15s on ability lung cancer cells to cross an endothelial monolayer (transendothelial migration).

5- To examine the effect(s) of knockdown and overexpression levels of FUT4 and FUT7 on the expression levels of CD15 and CD15s downstream.

6- To study the effect of knockdown and overexpression of CD15/FUT4 or CD15s/FUT7 on the adhesion ability of cancer cells on brain endothelia monolayer ability of cancer cells through brain endothelium monolayer.

7- To study the effect of knockdown and overexpression of CD15/FUT4 or CD15s/FUT7 on the transendothelial migration potential of cancer cells via the brain endothelium.
Objectives

The aims will be conducted via:

1- Immunocytochemistry (ICC), flow cytometry (FC), Western blotting (WB) and immunohistochemistry (IHC) (in human fixed tissue sections). These techniques will be used to localise and characterise the expression of CD15, CD15s and CD62E in the cell lines below:

- SC-1800 (adult astrocytes non-neoplastic)
- hCMEC/D3 (brain microvascular endothelial cells)
- COR-L105 and A549 (Primary non-small cell lung cancer, NSCLC)
- SEBTA-001 and SEBTA-005 (metastatic NSCLC obtained from brain)
- NCI-H1299 (metastatic NSCLC obtained from lymph node)
- UP-007 and SNB-19 (GBM)

2- Immunocytochemistry (ICC), flow cytometry (FC) and Western blot (WB) will be conducted to investigate the expression of CD62E on activated brain endothelial cells at different concentrations of TNF-α. In parallel, cells will be stimulated with TNF-β as well to assess specificity of CD62E/ TNF-α.

3- A fluorometric qualitative and a quantitative cell-cell adhesion assay will be employed to examine the role of CD15 and CD15s on adhesion ability of NSCLC cells. This will be conducted under both static and shear stress conditions using Cellix microfluidics and live cell imaging with and without immunoblocking via anti-CD15 and anti-CD15s monoclonal antibodies.

4- The role of CD15 and CD15s in cancer cell transendothelial migration potential with and without immunoblocking via anti-CD15 and anti-CD15s monoclonal antibodies will be assessed via:
• EVOM™ (Voltohmeter)
• ECIS™ (Electric Cell-Substrate Impedance Sensing)
• Cellzscope® (Automated transendothelial resistance monitoring system).

5- The effect of genetically manipulating FUT4 and FUT7 on the expression of CD15 and CD15s respectively, will be assessed by, ICC, FC analysis and WB.

6- The effect of knockdown and overexpression of FUT4/CD15 and FUT7/CD15s on the adhesion of cancer cells will be determined by cell-cell adhesion assay.

7- The effect of knockdown and overexpression of FUT4/CD15 and FUT7/CD15s on the transendothelial migration ability of cancer cells via an endothelial monolayer will be assessed by EVOM™, ECIS™ and Cellzscope®.
CHAPTER TWO

MATERIALS AND METHODS
2.1 Cell culture and cell lines

All cell culture work was carried out aseptically in a sterile laminar flow cabinet (NUAIRE™ Biological safety cabinet-class II). All plasticware and glassware used for tissue culture were sterilised by autoclaving (121°C under 1.1 Bar G for 20 mins). All cell lines used within the scope of this project were of human origin. Cell lines were cultured in each of their appropriate media supplemented with different concentrations of heat-inactivated human serum (Sigma, UK) as described in Table 1. Cells were passaged into new T25 or T75 cell culture flasks (Greiner Bio One, UK) every 3-5 days by washing with Hank’s balanced salt solution (HBSS) (Invitrogen, UK) followed by harvesting using TrypLE™ (Invitrogen, UK). All cell cultures were maintained in a humidified incubator (NUAIRE DH AUTOFLOW CO2 Air-Jacketed) supplemented with 5% CO₂ and 95% air at 37°C.

2.1.2 Establishment of Primary cultures

Human tumour biopsies were obtained from excised gliomas or from brain metastatic tumours of patients from King’s College Hospital (London, UK), with Ethics permission 11/SC/0048. Biopsies were placed in sterile Petri dishes, washed with HBSS then dissected into homogenised small explant suspensions which were then transferred into DMEM+1% Penicillin/Streptomycin supplemented with 20% fetal calf serum (FCS) then gradually changes to medium supplemented with human serum in T12.5 tissue culture flasks and left to adhere for a week in a humidified incubator at 37°C and 5% CO₂ (Table 3).
<table>
<thead>
<tr>
<th>Cell line ID</th>
<th>Histological grading and characteristics</th>
<th>Donor’s gender</th>
<th>Media + serum supplementation used</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-1800</td>
<td>Astrocytes (non-neoplastic) <em>ScienceCell™ Research Laboratories, UK</em></td>
<td>Male</td>
<td>ABM+ AGM-2+ 3% HS</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>Immortalized cerebral microvascular endothelial cells (SV 40 transfected) Donated by Professor Pierre O. Couraud (Institute of Cochin, Paris, France)</td>
<td>Male</td>
<td>EBM+ EGM-2+ 2% HS</td>
</tr>
<tr>
<td>COR-L105</td>
<td>Established cell line; Lung adenocarcinoma <em>Sigma, UK</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>A549</td>
<td>Established cell line; lung adenocarcinoma <em>Sigma, UK</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>SEBTA-001</td>
<td>Low passage; metastatic lung carcinoma to the brain <em>In-house, biopsy-derived</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>SEBTA-005</td>
<td>Low passage; metastatic lung carcinoma to the brain <em>In-house, biopsy-derived</em></td>
<td>Female</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>NCI-H1299</td>
<td>Established cell line; metastatic lung carcinoma to cervical lymph nodes <em>Sigma, UK</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>UP-007</td>
<td>Low passage; heterogeneous Grade IV GBM <em>In-house, biopsy-derived</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
<tr>
<td>SNB-19</td>
<td>Established cell line, Grade IV GBM <em>DSMZ, Germany</em></td>
<td>Male</td>
<td>DMEM+10% HS</td>
</tr>
</tbody>
</table>

Table 3: Primary cell cultures and cell lines used which show the histological grading and characteristics of cells used in the scope of this thesis, donor’s gender and age. ‘UP’ stands for University of Portsmouth and ‘SEBTA’ for South of England Brain Tumour Alliance. These cultures were established ‘in-house’ from biopsies received from surgical resections. Growth medium and serum concentration used to maintain cell cultures throughout the project are also mentioned.
2.1.3 Cryopreservation of the cell lines

Recovery™ cell culture freezing medium (Invitrogen, UK) was used which was supplied complete with a dehydrating agent; dimethlysulfoxide (DMSO), which reduces the water content of the cells thus reducing ice crystal formation and therefore preventing damage to cell membranes. Briefly, cells were harvested as previously described, centrifuged and re-suspended in 1mL of cell culture freezing medium before transferring to cryotubes (Greiner, Bio-One, Germany). The cryotubes were placed in a freezing container which containing isopropanol to allow cells to be cooled at a rate of 1°C/minute and kept at -80 °C freezer overnight. The cryotubes were immersed in liquid nitrogen at -196 °C and stored for an indefinite period of time.

2.1.4 Resurrection of cells from liquid nitrogen stores

Cells were removed from the liquid nitrogen tank, thawed in a water bath at 37°C. The contents were transferred drop-wise to T25 flasks with 6mL of fresh pre-warmed medium (Table 3). Media was changed after incubating overnight to remove the cryopreserving chemical: dimethlysulfoxide (DMSO). Flasks were then returned to the incubator and maintained as previously described.
2.1.5 Cell counting

Two types of automated cell counter ViCell\textsuperscript{XR} Cell Viability Analyzer (Beckman Coulter, UK) and Countess II FL\textsuperscript{®} (Invitrogen, UK) were used to determine cell viability using the trypan blue exclusion assay (Strober, 2001). Trypan blue is taken up passively by dead cells while live cells with intact cell membrane exclude the dye. To perform a cell count, harvested cells were re-suspended in 1mL of growth media from which 10 µL of cell suspension removed and mixed with 10µL of trypan blue solution stain (Invitrogen, UK). 10µL of this cocktail was then mounted on Countess II FL\textsuperscript{®} slides (Invitrogen, UK) and slotted in the machine for the cell count to be carried out. To calculate the required volume of cell suspension that contained the required cell count, the following equation was used (Maherally, 2008):

\[
\frac{(1000) \times \text{number of cells required}}{\text{Total number of viable cells counted}} = \text{Required volume (µL) of cell suspension}
\]

(1000) X (number of cells required)

_________________________________________________________ = Required volume (µL) of cell suspension

Total number of viable cells counted
2.1.6 Growth curves and cell population doubling time

Most of the rapidly dividing cells grow in a sigmoid growth curve form (s-shape) starting with the lag phase then log or exponential phase and the saturation phase (plateau). To create a growth curve, cells were harvested and subcultured in T25 flasks at different seeding densities starting with: $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^5$ cells in triplicate. Cells were incubated for 24 hours then counted. Sampling and counting were repeated every 24 hour until a plateau was reached. Cell densities were plotted against time and population doubling time calculated as in Figure 12. Doubling time was calculated according the following equation: doubling time ($DT$) = $T \ln 2 / \ln (X_e/X_b)$

$T$: incubation time, $X_b$: cell number at the beginning of incubation time, $X_e$: cell number at the end of incubation time, (Growth curves of the studied cells: appendix 2).

Figure 12: Growth curve for cell culture

Figure 12: A sigmoid growth curve shows the growth phases of a cell culture which is comprised of lag phase while cells are adapting to the new environment and adhering followed by exponential phase which is a period of rapid growth and stationary phase where cells growth rate stabilises.
2.1.7 Cell population doubling time (PDT)

It is important to determine the population doubling time of the cell lines before starting the experiments as it helps to estimate the initial seeding density. To determine the population doubling time for each cell line, increase in viable cell number was recorded and plotted against time on a semi-log scale.

2.2 Mycoplasma test

All cell lines were tested every two weeks using MycoAlert™ Mycoplasma detection Kit (Lonza, UK). This test is based on a biochemical reaction to detect active mycoplasmal enzymes in cell culture supernatant. In principle, MycoAlert™ (Lonza, UK) substrate is catalyzed by viable mycoplasma enzymes in which ADP converted to ATP; and in turn luciferase enzyme in MycoAlert™ reagent (Lonza, UK) reacted with ATP is to produce a light signal (chemiluminescence). Briefly, cell culture medium supernatant was collected and centrifuged at 1000rpm for 5 minutes then; 25µL of the supernatant was mixed with 25µL of MycoAlert™ reagent in a solid white, F-bottom, 96-well plate. Plates were incubated at room temperature for 5 minutes then, reading-A was taken using a POLARstar microplate reader (BMG, UK); then 25µL of MycoAlert™ Substrate was added, followed by incubation for 10 minutes at room temperature and reading-B was taken; results were analysed by dividing reading-B value on reading-A and samples with a ratio lower than one considered mycoplasma free, while results higher than one referred to a contamination with mycoplasma.
2.3 Flow cytometry (FC)

Flow cytometry was conducted by harvesting cells with gentle scraping, collected in 1mL of complete medium and centrifuged at 1000 rpm for 5 minutes using a 5415R microfuge (C&M Scientific Ltd, UK). Samples were re-suspended in 1mL of phosphate buffered saline (PBS) (Sigma Aldrich, UK) and split into five eppendorf tubes as follows: one negative control (cells resuspended in PBS only), one isotype control to ensure primary antibodies specificity and efficiency and three positive samples (Table 4). Samples were incubated with 100µl of specific primary antibody for 30 minutes at 4°C. This step was omitted for the negative control. Samples were centrifuged, supernatant discarded and pellet suspended in 1mL of PBS+2% Bovine serum albumin (BSA) (Sigma Aldrich, UK), followed by incubation with 200µL of secondary antibodies at a dilution of 1:100 for 30 minutes at 4°C (Table 6). Samples were washed twice before pellets were suspended with 500 µL of PBS then transferred to FACS tubes (BD Biosciences, UK). 5µL of Propidium Iodide (PI) (Sigma Aldrich, UK) was added to all the samples to stain the nuclei of dead cells thus excluding them from the gated cell count. Each sample was tested in triplicate (negative control + isotype control + 3 positives) in at least three independent experiments. Samples were analysed using a dual-laser FACS Calibur (BD Biosciences, UK) designed for multicolour analysis, equipped with a 488 nm Argon gas laser and a 635 nm red diode laser. Acquisition and analysis were conducted via Pro software.
### 2.3.1 Statistical analyses of flow cytometry results

Results were statistically analysed using one-way ANOVA followed by Tukey’s multiple comparison post-test with a probability of less than 0.01 was regarded as a significant using Graph Pad Prism 3.02 software. N=3.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
<td>1:10</td>
<td>Sigma, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgM</td>
<td>1:100</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>CD15s</td>
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<td>Goat anti-mouse Alexa Fluor 488 IgG</td>
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<tr>
<td>IgM isotype control</td>
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<td>Goat anti-mouse Alexa Fluor 488 IgM</td>
<td>1:100</td>
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<td>IgG isotype control</td>
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<td>Invitrogen, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgG</td>
<td>1:100</td>
<td>Invitrogen, UK</td>
</tr>
</tbody>
</table>

Table 4: Primary and Secondary Antibodies used for Flow cytometry (FC). The table shows the primary, secondary and isotype control antibodies with their corresponding species and dilutions which were used for flow cytometry.
2.4 Immunocytochemistry (ICC)

Cells were harvested from 80% confluent tissue culture flasks and seeded onto 8 mm sterile coverslips (UKEG, UK) at 1x10⁴/well and incubated overnight at 5% CO₂ and humidified atmosphere at 37°C and allowed to reach 70-80% confluency. Cells were fixed with 4% paraformaldehyde (PFA), pH 7.4 (Sigma Aldrich, UK) for 3 minutes followed by three washes with PBS then blocking with 10% normal serum (species dependent on secondary antibody used) (Sigma Aldrich, UK) followed by incubation with primary antibody for one hour and secondary antibody for 30 minutes at room temperature. Nuclei were counterstained with 1µg/mL Hoechst Blue solution (HB) (Sigma Aldrich, UK) for 30 seconds. Coverslips were mounted on slides using Vectashield® (Vector Laboratories, UK). Washes with PBS (5 minutes x 3) were carried out prior and post every incubation step. Where Multi-labelling was needed, antibodies were cocktailed (raised in different species host) for both antibody incubation time. No primary antibody was added to negative control or isotype control. An isotype control was used to ensure primary antibodies specificity and binding efficiency (Table 5).
<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
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<td>Sigma, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgM</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>10% goat serum</td>
</tr>
<tr>
<td>CD15s</td>
<td>1:50</td>
<td>Millipore, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgM</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>10% goat serum</td>
</tr>
<tr>
<td>CD62E</td>
<td>1:500</td>
<td>Sigma, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgG</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>10% goat serum</td>
</tr>
<tr>
<td>IgM isotype control</td>
<td>1:100</td>
<td>Invitrogen, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgM</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>10% goat serum</td>
</tr>
<tr>
<td>IgG isotype control</td>
<td>1:100</td>
<td>Invitrogen, UK</td>
<td>Goat anti-mouse Alexa Fluor 488 IgG</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>10% goat serum</td>
</tr>
</tbody>
</table>

Table 5: Primary and secondary antibodies used with their corresponding blocking buffers for ICC. The table shows the primary, secondary and isotype control antibodies with their respective dilution and blocking buffers.
2.5 Immunoblotting

A semi-quantitative analysis was demonstrated to measure the level of expression of target antigen in cell lysate.

2.5.1 Preparing cell membrane lysate

Cell cultures were grown to reach 80-90% confluency. To lyse, growth medium was removed and cells were washed three times with 5mL ice-cold phosphate buffer saline (PBS) (Sigma Aldrich, UK), the subcellular protein fractionation kit was used (Thermo-scientific, UK) following manufacturer’s instructions and cellular compartments were sequentially extracted using variant lysis buffers from the kit. First, cells were incubated in cytoplasmic extraction buffer (CEB) for 10 minutes at 4 °C then a cell pellet was collected in cold eppendorf tubes and centrifuged at 2893 rpm for 5 minutes at 4 °C using 5418R centrifuge (Eppendorf, UK); the supernatant was removed (cytoplasmic extract) and cell membrane extraction buffer (MEB) was added to the remaining pellet followed by incubation for 15 minutes at 4°C with frequent vortex on the highest speed then, centrifuged at 7085 rpm for 5 minutes at 4°C, the supernatant (membrane extract) was collected and stored at -20°C.

2.5.2 Bicinchoninic Acid Protein Assay (BCA)

The BCA assay was used to quantify the amount of total protein in the concentration range 0.2-1.0 mg. This assay relies on the formation of Cu²⁺-protein complex at alkaline conditions and reduction of Cu²⁺ to Cu¹⁺ which coordinates the amount of protein in the solution with formation of purple-blue colour complex. Colour density indicated the reduction of Cu²⁺ (Smith et al., 1987). Different concentrations of BSA (0-2000 µg/mL) were prepared from BSA stock solution 2 g/mL (Sigma Aldrich, UK) and used as standards. BCA working solution stock was prepared by mixing 50 parts of reagent A (bicinchoninic acid, sodium tartrate, sodium carbonate, sodium bicarbonate in 0.1 N
NaOH, pH=11.25) (Sigma, UK) with 1 part of reagent B (4% w/v CuSO₄·5H₂O) (Sigma, UK). BCA working solution was added to BSA-standards and samples at ratio 20:1 respectively, in 96-Well Plate (Thermo Scientific, UK). Plates were then incubated for 30 minutes at room temperature before absorbance was measured by POLARstar microplate reader (BMG, UK) at a wavelength between 540-590 nm. A standard curve was plotted and its equation was used to calculate the total amount of protein in unknown samples.

2.5.3 Western blotting (WB)

WB was performed using cell membrane extracts which were isolated using the cell fractionation kit and the total amount of protein was quantified by BCA assay (2.7.2). Cell lysates were separated through gel electrophoresis using a 10% acrylamide SDS-PAGE gel cassette (8cmx10cmx5mm) (Thermo Scientific, UK) at room temperature for 90 minutes at 100 volt/ 400 Watt then samples were transferred (wet transfer) to a polyvinylidene fluoride (PVDF) (Bio-Rad, UK) membrane for 90 minutes followed by blocking with 5% skimmed powdered milk (Marvel, UK) or 5% BSA (Sigma Aldrich, UK) in Tris buffered saline (TBS) (150 mM NaCl, 10mM Tris, pH 7.4 with 0.05% Tween 20) (Sigma, UK) for an hour at room temperature. Primary antibody incubation was carried out overnight at 4°C as shown in table 6, followed by applying the secondary antibody horseradish peroxidase-conjugates. Extensive washing of the membranes was carried out in TBST (Thermo Scientific, UK) after incubation and immunocomplexes were revealed using an enhanced chemiluminescence reagent (Millipore, USA). Blots were visualised using a GBOX Chemi XT16 system (Syngene, UK).
<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
<td>1:500</td>
<td>Sigma, UK</td>
<td>Goat anti-mouse (HRP)</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>5% skimmed powdered milk</td>
</tr>
<tr>
<td>CD15s</td>
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<td>Millipore, UK</td>
<td>Goat anti-mouse (HRP)</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>5% BSA</td>
</tr>
<tr>
<td>CD62E</td>
<td>1:500</td>
<td>Sigma, UK</td>
<td>Goat anti-mouse (HRP)</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>5% skimmed powdered milk</td>
</tr>
<tr>
<td>ABCE1</td>
<td>1:500</td>
<td>Novus, UK</td>
<td>Goat anti-mouse (HRP)</td>
<td>1:500</td>
<td>Invitrogen, UK</td>
<td>5% skimmed powdered milk</td>
</tr>
</tbody>
</table>

Table 6: Primary and secondary antibodies used with their corresponding blocking buffers and respective dilutions and blocking buffers used for Western blotting.
2.6 Immunohistochemistry (IHC)

IHC was performed on human tissue sections kindly provided by Dr Federico Roncaroli, Institute of Brain Behaviour and Mental Health, The University of Manchester. IHC was conducted on paraffin embedded, formalin fixed tissue sections (FFPE) of human lung to brain metastatic cancer biopsies using antibodies to CD15, CD15s and CD62E (Table 7). Briefly, 4μm thick FFPE sections were dewaxed in Xylene then, rehydrated with 100%, 95%, 70% Ethanol and deionized water respectively, followed by 40 minutes of heat-induced epitope retrieval with citrate buffer (6.0 pH) at 95°C then incubated for 30 minutes with 3% hydrogen peroxide in methanol to block endogenous peroxidase and biotin activity. Tissues were blocked for 30 minutes with 3% horse serum and probed with primary antibodies as shown in table 5 for one hour at room temperature. The Elite Vector Stain ABC system (Vector Labs, UK) was employed as a detection system and DAB stain (Vector Labs, UK) was used as a chromogen. Meyer’s haematoxylin (Sigma Aldrich, UK) was used as a counterstain. Mouse IgM isotype was used instead of primary antibodies in negative controls. Tissue sections were examined using a bright field automated microscope Ariol (Leica, Germany) for qualitative image analysis.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
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<td>Dako, USA</td>
<td>3% horse serum</td>
</tr>
<tr>
<td>CD15s</td>
<td>1:100</td>
<td>Millipore, USA</td>
<td>3% horse serum</td>
</tr>
<tr>
<td>CD62E</td>
<td>1:200</td>
<td>Sigma, UK</td>
<td>3% horse serum</td>
</tr>
</tbody>
</table>

Table 7: Primary, secondary antibodies and blocking buffers used for IHC. The table shows the primary antibodies and their respective dilutions and buffers used for IHC.
2.7 Confocal Microscopy

Confocal images were obtained using the X40 and X100 (oil immersion) objectives of a Zeiss LSM 510 Meta Axioskop2 confocal microscope using four lasers with excitation wave lengths of 405nm (blue), 488nm (green), 568nm (red) and 674 (purple), with diode, argon and HeNe1 lasers respectively. Microscopic images were obtained using optimized setting of pinhole diameter, offset acquisition and signal gain to lower the background noise. Two-dimensional (X-Y) and 3-dimansional (X,Y and Z-stack) Multi track images were captured with four separate channels with different colors, to avoid possible overlapping in excitation spectra. Samples stained with isotype control antibodies were used to set up the optimal setting.

2.8 Live cell imaging (Real-time Kinetic Microscopy)

A live cell imaging microscope consisting of an inverted Zeiss Axiovert 200M live cell (time-lapse) microscope with a modified stage, which is temperature controlled by an external heating sensing system and CO₂-controlled, operated by a CO₂ concentration sensing unit connected to a regular cylinder of pure CO₂ gas was used to study the cellular behaviour of cancer cell-hCMC/D3 co-culture in real time. hCMC/D3 (7.5x10⁴/well) were plated in 24-well plates (Corning, UK) and incubated overnight to form a monolayer. Cancer cells (1.0x10⁴/well) were tagged with green fluorescent live cell tracker (Abcam, UK) and seeded on top of the hCMC/D3 monolayer. The Zeiss Axiovert 200M live cell (time-lapse) microscope was then set up to image three different points in each well, once every 30 minutes over 48 hours. Two types of filters were used to capture the images: DAPI filter for the brightfield and FITC filter set for green-tagged cells or GFP transfected cells. Images were collated and a movie sequence generated using Volocity 6 software.
2.9 Genetic modification of FUT4 and FUT7 (stable transfection)

CD15/CD15s are trisaccharide epitopes formed by fucosylation of the polylactosamine chain via α1,3-fucosyltransferase (α1,3-FUT4) enzyme (Lowe et al., 1991). It was revealed that a high level of CD15 was correlated with the overexpression of FUT4 enzyme due to transfection with FUT4 gene (Clarke 1996, Kudo et al., 1998, Kaneko et al., 1999 Nakayama et al., 2001). It has been previously reported that upregulation of FUT7 gene led to overexpression of CD15s (Sasaki et al., 1994, Natsuka et al., 1994, Marer et al., 1997, Kimura et al., 1997 and Nakayama et al., 2001) and FUT4 knockdown has been shown to decrease the expression of CD15 (Lowe and Marth, 2003 and Allahverdian et al., 2010, Weston et al., 1999 and Allahverdian et al., 2010). Thus, FUT4 and FUT7 gene were chosen to regulate the extracellular expression of CD15 and CD15s respectively. Four different human cell lines were transfected: lung-brain metastatic cancer (SEBTA-001), lung-lymph node metastatic cancer (NCI-H1299), primary lung cancer (COR-L105) and GBM (UP-007). Transfection was carried out by liptofection using TurboFection 8.0 (Origene, USA) and DNA retroviral GFP vectors (Origene, USA).

2.9.1 Up-regulation (overexpression) of FUT4/CD15 and FUT7/CD15s

To enhance the extracellular expression of CD15 and CD15s, target cells were transfected with cDNA of the FUT4 gene and the FUT7 gene respectively by introducing a full length cDNA of FUT4 (GeneBank accession number: M58596) and FUT7 (GeneBank accession number: U11281) (Origene, UK). cDNA constructs were cloned into a retroviral GFP vector: pCMV6-AC-GFP by the manufacturer and were ready for immediate use (commercially available from; Origene, USA) (Appendix 3).
2.9.1.1 Transfection with cDNA of *FUT4* and *FUT7*

Prior to transfection, within 24 hours $1 \times 10^5$ of target cells (SEBTA-001, NCI-H1299, COR-L105 and UP-007) were seeded onto sterile 12-well tissue culture plate in full growth medium, to obtain 50 to 70% confluency. On the day of transfection, cDNA complex was prepared by adding 100µL of Opti-MEMI® serum free medium (Gibco, UK) into sterile plastic micro-tubes then 4 µL of TurboFection 8.0 (Origen, USA) followed by gentle mixing by re-pipetting, the mixture was incubated for 5 minutes, then 3 µg/well of dd H$_2$O diluted plasmids were added to the complex followed by 30 minutes incubation. Meanwhile, the old medium was removed from plated cells and replaced by 2 mL of fresh Opti-MEMI® serum free medium. cDNA complex was added in a drop wise manner. The plate was rocked gently to distribute the complex evenly. Transfected cells were incubated for 48 hours then cells were passaged at a dilution of 1:10 into 24-well plates in full growth medium (DMEM+10% FCS) containing 1000µg/mL of neomycin (G 418) as a selective medium. A mock transfection well (vehicle) was applied using non-coding pCMV6-AC-GFP tagged plasmid (PS 1000010) (Origen, USA), for each cell line in parallel with the positive transfection and wild type control well.
2.9.1.2 cDNA vectors and constructs

- **FUT4- human cDNA ORF clone**: pCMV6-AC-GFP retroviral GFP vector ready-cloned, contained a unique construct of alpha (1, 3) fucosyltransferase, a myeloid-specific protein (FUT4), a CMV promoter, a T7 promoter, a G418 resistance gene and a C-terminal TurboGFP tag (Appendix 3) (Origene, USA).

- **FUT7- human cDNA ORF clone**: pCMV6-AC-GFP retroviral GFP vector ready-cloned: which included a unique construct of alpha (1, 3) fucosyltransferase (FUT7) protein, a CMV promoter, a T7 promoter, a G418 resistance gene and a C-terminal TurboGFP tag (Appendix 3) (OriGene, USA).

2.9.2 Knockdown of FUT4 and FUT7

FUT4 and FUT7 genes were knockdown in the following cell lines: UP-007, SEBTA-001, NCI-H1299 and COR-L105 by transfecting cells with four different human-FUT4 and FUT7 unique 29mer shRNA constructs in pGF-V-RS GFP vectors, all interfering plasmids were designed according to the sequence of the human FUT4 gene (GeneBank accession number: M35531) and FUT7 gene (GeneBank accession number: U11281), designed using the siRNA-designing program (Ambion Inc). All constructs were cloned into the vectors and ready for immediate use (OriGene, USA). Target cells were seeded in 12-well plates in serum supplemented growth medium (DMEM+10% FCS) (Gibco, UK) for 24 hours to reach 50-70% confluency. Cells were washed twice followed by addition of 2 mL of serum free medium Opti-MEMI® serum free medium (Gibco, UK). To prepare the shRNA complex, 50mL of sterile ddH2O was added to dilute the shRNA then mixed by gentle vortexing. 3µg of shRNA was added to 250 µL of Opti-MEM® serum free medium (Gibco, UK) in sterile plastic micro-tubes and mixed with 3µL of TurboFection 8.0 (OriGene, UK). The complex was mixed by pipetting. Tubes were then incubated for 30 minutes at 37°C. The developed transfection complex was added to target cells, in a drop wise manner.
and rocking for an even distribution. The transfected cells were incubated for 48 hours followed by harvesting and seeding at 1:10 dilution in DMEM+10%FCS+ 1000 µg/mL G418 as a selection medium.

2.9.2.1 shRNA vectors and constructs

- **FUT4-** human, 4 unique 29mer shRNA constructs in lentiviral GFP vector ready-cloned (TG312897). This kit was comprised of one set of four unique constructs of shRNA ready-cloned under U6 promoter for mammalian cells, a T7 promoter, a G418 resistance gene and a C-terminal TurboGF tag (Origene, USA).

  **FUT4 shRNA constructs**
  1- AGGAGGTGGATCTCGCGTGTTGGACTAC (GI351581)(Origene, USA)  
  2- TCGGAAGCTGGTGGCAAGTACTCTCTTTCAAC (GI351582)(Origene, USA)  
  3- TTCGAGAAGCTCGACCTGGATTATAT (GI351583)(Origene, USA)  
  4- AGCTACGCTGGACTCGCATACCTCTCTCTCT (GI351584)(Origene, USA)

- **FUT7-** human, 4 unique 29mer shRNA constructs in retroviral GFP vector ready-cloned (TG312894). This kit was comprised of one set of four unique constructs of shRNA ready-cloned under a U6 promoter for mammalian cells, a T7 promoter, a G418 resistance gene and a C-terminal TurboGF tag (Origene, USA).

  **FUT7 shRNA constructs**
  1- TCAGCCACCTGGAGGACCTCTCCACTGG (TG312897A)(Origene,USA)  
  2- TGAATGAGCCGATACCAACGCTTCTT(TG312897B) (Origene,USA)  
  3- TCTACCTGTCTTTTGAGAACTCTCACTAC (TG312897C)(Origene,USA)  
  4- CCACGATCCACATCCACTCTCTCTCTCTC (TG312897D) (Origene,USA)
2.9.3 Determining the optimal concentration of selection marker (G418)
Optimisation is a critical step in stable transfection. The transfected cells were positively selected using G418 as a selection marker. The optimal concentration of G418 was determined for each cell line by generating a kill curve which is a dose-response experiment. 1x10^3 cells were seeded on sterile 96-well plates and cells were subjected to increasing doses of G418 (0μg/mL-1300μg/mL). The viability of cells was tested using an MTS assay described in 2.12 every 24 hours over one week. The optimal concentration was the lowest G418 concentration at which all cells were non-viable after one week of incubation in selecting medium.

2.9.4 Selection of stable transfected cells
It was necessary to establish a stable transfected cell line to assess the transfection efficiency and to ensure the reliability of the functional studies. Stable transfected cells of FUT4 and FUT7 (overexpressing and knockdown) were established and used within the scope of this project. After 48 hours post-transfection, cells were passaged into 1:10 and transfection medium was replaced with growth medium with the optimal concentration of selection marker G418 (Geneticin) (1100 μg/mL). The optimal concentration was determined according to the cell line kill curve as in (2.11.3). One week after, the cells were transferred in 48-well plates, grown in growth medium to expand the number of transfected cells followed by assessment of expression when cells were preserved in liquid nitrogen for further investigation.
2.10 Cell viability assay (MTS)

The colourimetric CellTiter 96® Aqueous one solution cell proliferation assay (MTS) (Promega, UK) was used to assess the viability of cells under study. The CellTiter 96® Aqueous one solution Reagent works by reducing the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in viable cells to formazan products as shown in Figure 13. Briefly, cells were seeded at 1 x 10^4 in 96-well plates and incubated at 37°C and 5% CO₂ for 24 hours. 0.33mg/mL of CellTiter 96® Aqueous one solution cell proliferation reagent (MTS) was added to wells and incubated for 3 hours. Absorbance was measured at 490 nm using a POLARstar™ OPTIMA microplate reader (BMG, UK).

2.10.1 Statistical analyses for viability

All experiments were performed 3 times in triplicate unless stated otherwise. Data is expressed as +/- SE. Statistical analyses were performed using One-way ANOVA followed by Tukey’s multiple comparison post-hoc tests. Graph Pad Prism 6 software was used for analysis. Results were considered significant when *p<0.05, **p<0.01 and ***p<0.001. N=3

Figure 13: Mechanism of action of MTS

Figure 13: Mechanism of action of MTS. It shows the electron transition (reduction) in the MTS solution to Formazan achieved by an intermediate electron acceptor from NADH in viable cells
2.11.1 Quantitative adhesion assay (under static conditions)

The CytoSelect™ Tumour-Endothelium Adhesion Assay Kit (Cell Biolabs, UK) was used to evaluate the adhesion potential of tumour cells on the brain endothelial cell monolayer, following an amended version of the manufacturer’s protocol (Jassam et al, 2015) (Figure 14). 1x10^6 cells/well of hCMEC/D3 were seeded onto a 96-well plate pre-coated with fibronectin (10µg/mL) and grown to reach 80-90% confluency. The endothelial cell monolayer was first treated with 25pg/mL of TNF-α overnight to activate the extracellular expression of CD62E. Primary and secondary lung cancer cell lines were then tagged with a green fluorescent dye (Cyto Tracker™) (Cell Biolabs, UK). 1x10^5 cancer cells were then seeded on top of the activated hCMEC/D3 monolayer for 90 minutes. Non-adherent cells were washed off with pre-warmed PBS, the whole co-culture was lysed using 2x lysing buffer (Cell Biolabs, UK) and the relative number of adherent cancer cells was quantified by reading the plates on fluorescence plate reader at 485nm/538nm filter set and 530 cut-off using a POLARstar™ OPTIMA microplate reader (BMG, UK). The experiment was repeated at least three times in triplicate.

2.11.2 Qualitative adhesion assays

To qualitatively evaluate adhesion, ICC and confocal image analysis was used. 1x10^6 cells/well of hCMEC/D3 were seeded on top of 8mm sterile glass coverslip pre-coated with fibronectin (10µg/mL). Cells were incubated in complete growth medium supplemented with 25pg/mL of TNF-α to reach confluency. 1x10^5 of green fluorescently-tagged cancer cells were added on an hCMEC/D3 monolayer and incubated for 90 minutes. Non-adherent cancer cells were washed away with pre-warm HBSS. Cells were fixed with 4% PFA and counterstained with HB. Coverslips were mounted on slides and examined using a Zeiss confocal microscope. Semi-quantification of adhesion was assessed using confocal images and Zeiss ZEN software (Figure 14).
Figure 14: Cancer cell-brain endothelium adhesion assay

Figure 14: Cancer cell-brain endothelium adhesion assay. Schematic shows the protocol of cancer cell-brain endothelium assay, 96-well plates were pre-coated with fibronectin (10µg/mL) and hCMEC/D3 cells at 1x10^6 cells/well were seeded and grown to confluency, endothelial cells were activated with 25pg/mL of TNF-α overnight. NSCLC cell were tagged with a green fluorescent dye (Cyto Tracker™) cancer cells and then 1x10^5 cells were applied on top of the hCMEC/D3 monolayer and the co-culture was incubated for 90 minutes. Non-adherent cells were washed off using pre-warmed PBS, the co-culture was lysed using 2x lysing buffer and the relative number of adherent cancer cells was quantified by reading the plates on fluorescence plate reader at 485nm/538nm filter set and 530 cut-off using a POLARstar™ OPTIMA microplate reader, results were validated with qualitative adhesion assay by using the confocal microscope (Jassam et al., 2016). The experiment was repeated at least three times in triplicate.
2.11.3 Cancer cell adhesion (under shear stress)

A microfluidic assay was carried out by seeding endothelial cells in Vena8 Endothelial™ biochips (channel volume: 2.69µL) (CellixLtd, UK). The biochips were pre-coated with 0.5mm of 10µg/mL fibronectin solution (Sigma, UK) and incubated for one hour. 1.5x10^6 of hCMEC/D3 cells were seeded in each channel and incubated for 2 hours. The biochips were then connected to a Microfluidic pump (CellixLtd, UK) and the whole unit was kept overnight in an incubator at 37° C, 5% CO₂ under shear stress [flow on perfusion mode with a 15mL/hr volumetric flow rate (2.5 dyn/cm²)]. The biochip was connected to a Zeiss Axiovert 200M inverted live cell (time lapse) (as detailed in 2.10) microscope and cancer cells (green fluorescently-tagged) at 1x10^6 cells/mL were then pumped onto the hCMEC/D3 monolayer at 2.5 dyn/cm² controlled by a Mirus Evo nanopump (CellixLtd, UK) and analyzed via Vena Flux Assay software. Live cell images were taken once every 10 minutes over 72 hours to monitor cancer cell adhesion on the hCMEC/D3 monolayer. The images were collated and movie sequences generated using Volocity software V5.4. The experiment was repeated three independent times in triplicate.

2.11.4 Statistical analyses for adhesion assays

All experiments were performed 3 times in triplicate unless stated otherwise. Data is expressed as +/- SE. Statistical analyses were performed using One-way ANOVA followed by Tukey’s multiple comparison post-hoc tests, Graph Pad Prism 6 software was used for analysis. Results were considered significant when *p<0.05, **p<0.01 and ***p<0.001. N=3
2.12 Transendothelial migration assays

Endothelial cells are characterised by a unique feature which is the formation of a monolayer that are tightly connected with specific regions called tight junctions (Abbott et al., 2006) to form an intact barrier consistency of a continuous membrane of endothelial cells. One of the suggested and generally used methods was measuring the resistance of the barrier according to Ohm’s law. This method is based on the qualitative measurement of electric resistance of the barrier which reflects the cellular barrier integrity (Benson et al., 2013). In this method, the resistance of the monolayer is measured by applying direct alternating current (AC) (I) square wave with a frequency of 12.5 Hz with defined voltage (U). The resistance of the barrier can be calculated according to Ohm’s law: Resistance (R) = Voltage (U) / Current (I) (Benson et al., 2013). In this project, three methods were used to study the changes in the resistance of brain endothelial cells monolayer (hCMEC/D3) and used as a suggestive indicator on the ability of cancer cells to undergo transendothelial migration across the monolayer of brain endothelial cells.

2.12.1 Endothelial Voltohmmeter (EVOM)

EVOM was used to measure the resistance of the barrier formed by a continuous monolayer of endothelium cells grown on a porous membrane. EVOM (World Precision Instruments, USA) is a battery-operated device equipped with a set of STX2 two chopstick electrodes. One is silver/silver-chloride pellet to measure the voltage and the other is silver electrode for passing current. Before measurement, the EVOM was set on a resistance range of 2000 ohm and alternating current input. 24 well plate, polycarbonate membrane, 8.0μm pore size Transwell® inserts (Corning, UK) were pre-coated with 10μg/mL fibronectin (Sigma Aldrich, UK). Fresh medium [EBM+ EGM-2+ 2% HS + TNF-α (25pg/mL)] (Lonza, UK) was added in the insert and the 24-well plate then, 1x10⁵ of hCMEC/D3 cells were seeded onto the luminal (upper) part of the insert. Two hours post-seeding, EVOM electrodes were placed into the transwell chambers (Figure 14).
Five readings per transwell were taken three times a day for 7-9 days. Results were averaged and resistance values plotted against time. At the end of the experiment, cells in the transwell inserts were fixed using the fixing solution from the Diff quick staining kit (Thermo Scientific, UK). A cotton bud was used to remove cells from the luminal side of polycarbonate membrane of the insert and the basolateral side of the insert stained. To count transmigrated cells, inserts were examined using a bright field microscope. All experiments were done in triplicate and repeated at least three times (N=3).

Figure 14: Measurement of resistance of hCMEC/D3 monolayer using Endothelial Voltohmmeter (EVOM). It illustrates the measurement of the resistance of hCMEC/D3 monolayer using EVOM and the compartments of transwell-insert unit.

Figure 15: Measurement of resistance of hCMEC/D3 monolayer using Endothelial Voltohmmeter (EVOM)
2.12.2 Electric cell-substrate impedance sensing system (ECIS®)

ECIS was used to assess the effect of cancer cells on the integrity and resistance of brain endothelial monolayers at a two dimensional level. The ECIS® model 1600R, relay bank, lock-in amplifier and the software all were purchased from applied Biophysics, USA. ECIS is an automated real-time, label-free and impedance-based instrument for monitoring and recording different cellular activities of the barrier using non-invasive alternating current (AC) signals (Giaever and Keese, 1991). The measurement system consisted of 8-well cell culture dish array (8W10E+PC) (Ibidi, UK), equipped with a gold large counter and 40 small sensing electrodes per well with a diameter of 250µm (Figure 16). To set up the experiment, tissue culture dish arrays (8W10E+, PC) (0.9 cm² in basal area and 0.7 mL total volume) were washed with HBSS and activated with 10mM L-cysteine for 10 minutes at 37°C in an humidified incubator. Wells were washed with HBSS then coated with 10mg/mL Fibronectin for 2 hours followed by seeding of 75,000 hCMEC/D3 per well in 400µL of EBM+ EGM-2 +2% +TNF-α (25pg/mL). The arrays were then connected to the instrument, the electrodes (large and small) were connected by relay bank to a phase-sensitive lock-in amplifier then AC current was applied through a 1-MΩ resistor to monitor the formation of an intact endothelial cell monolayer. Once the resistance plateaued/reached the peak resistance, 20,000 of cancer cells were added on top of the endothelial cell monolayer. The confluency of the endothelial cell monolayer was evaluated by light microscopy imaging. The electrical resistance of each well was measured every 2.5 minutes. 16 wells were tested at the same time; time series data were collected and analysed using ECIS core software.
2.12.3 Impedance spectroscopy (CellZscope®)

The impedance spectroscopy technique was used to measure the transendothelial electric resistance (TEER) of monolayers of cells grown on porous membranes in standard tissue culture inserts (Figure 17). CellZscope® is an automated computer controlled system which is based on applying a non-invasive AC voltage and measuring the resistance of the monolayer barrier. In this project, all the system compartments including the cellZscope®, the external controller and the software were obtained from (nanoAnalytics, Germany). CellZscope® was mainly used to measure the resistance of brain endothelial cell (hCMEC/D3) monolayer barriers, formed by growing cells on 8µm pore size permeable membranes in transwells. The electric resistance of the cell monolayer barrier was measured by an electric equivalent circuit and data analysed as a TEER value via a corresponding mathematical model. To start the experiment, 24-well type cell module wells were cleaned with 70% ethanol (Sigma Aldrich, UK) then washed with HBSS and 800 μL of EBM+ EGM-2 +2% +TNF-α (25pg/mL) was added to the sample wells.
Pre-coated (fibronectin: 10µg/mL) transwell-inserts with a polycarbonate translucent membrane, 8µm pore size (Corning, UK) were placed in the sample well of the cellZscope module, then 1x10^5 hCMEC/D3 cells were seeded onto the inserts. The cellZscope module was placed in a sterile, humidified, 37°C and 5% CO₂ mini GalaxyE incubator (C and M Scientific) and connected to the cellZscope external controller, which in turn was connected to the cellZscope 1.5.0 software throughout a USB port. Once TEER values plateaued/reached a peak, 20,000 cancer cells were added on top of the brain endothelial cell monolayer. Readings of monolayer resistance and cell impedance were automatically monitored every 30 minutes. TEER values and histograms were collated and analysed using the same operating software.

**Figure 17: Measurement of transendothelial resistance by CellZscope®.**

![Schematic illustration](image)

Figure 17: Measurement of transendothelial resistance by CellZscope®. A schematic illustration shows equivalent circuit diagram of the measurement of resistance of hCMEC/D3 monolayer. Cells were grown on a porous 8µm translucent membrane. Non-invasive AC current passes through the monolayer of cells. Any disruption that occurs to the barrier is shown as a decrease in resistance value.
2.13 Synchronization of cell cultures

Expression of CD15 and CD15s were characterised in non-synchronised cell cultures in primary and secondary brain tumour cells as per the flow chart below (Figure 1). Cell cultures were synchronised at G1 phase by serum starvation, at S phase via Hydroxyurea (1mM) and at G2/M phase via Nocodazole (Timson 1975).

**G1 phase:** cells were arrested at G1 phase by serum deprivation. $1 \times 10^6$ cells were seeded in T25 tissue culture flasks containing serum supplemented growth medium until 50% confluency was reached. Cells were then washed with pre-warmed sterile Hank`s buffered salt solution (Fisher, UK) followed by addition of growth medium supplemented with 1% human serum followed by overnight incubation. Cells were then grown in serum-free medium for 48-72 hours then replaced every 12 hours to avoid cell cytotoxicity due to the pH change.

**S phase:** cells were arrested at S phase by first arresting cells at G1 phase then replacing the medium with growth medium supplemented with serum and Hydroxyurea (Sigma, UK) at a final concentration of 1mM and incubated overnight.

**G2/M phase:** cells were grown in serum-free medium for 24 hours followed by replacement with fresh growth medium supplemented with 2μg/mL Nocodazole (Sigma, UK). Growth factors in the medium induce cells to progress to G2/M phase while Nocodazole causes cell arrest at G2/M phase since Nocodazole depolarizes the tubulin in microtubules.
2.14 Detection of cell cycle stage

To determine the distribution of cell cycle stages, non-synchronized and synchronized cell cultures were harvested by gentle scraping. Cellular pellets were washed with PBS and fixed with ice-cold 70% Ethanol for 48 hours at 4°C. Fixed cells were washed with PBS+2% goat serum, resuspended in 250μL of Propidium iodide/RNase solution (FxCycle™) (Life technology, UK) and incubated for an hour at room temperature. Cells were then washed with PBS+2% goat serum and cell cycle analysis was conducted using a BD FACS Calibur (BD Biosciences, UK).

2.15 Immunocytochemistry using Premo™ (FUCCI) Cell cycle Sensor (BacMam 2.0)

A fluorescence ubiquitination cell cycle indicator (FUCCI) was used according to the manufacturer’s instructions (Life Technology, UK) to assess cell cycle progression. Cell cultures were transfected with the BacMam 2.0 gene delivery system which combines two main cell cycle regulators: Cdt1-tagged with red fluorescent protein (RFP) and geminin-tagged with green fluorescent protein (GFP). Cells in G0/G1 phase expressed Cdt1-tagged with RFP were visualised as cells with red nuclei in G0/G1 phase. Cells in S phase co-expressed Cdt1-RFP and geminin-GFP and were visualised with yellow nuclei. Geminin-GFP is predominately expressed in cells during G2/M phase allowing cells with green nuclei to be observed. Briefly, 1x10^3 BacMam2.0™ particles were diluted in 200μL serum free Opti-MEM™ (Gibco, UK) followed by addition of 1x10^3 cells and incubated for 10 minutes. Treated cells were seeded on 10mm sterile coverslips in 48-well plates followed by 48-hour incubation. Cells were fixed with 4% paraformaldehyde (Sigma, UK) and non-specific antigens were blocked with 10% goat serum (Sigma, UK). CD15 and CD15s primary antibodies were applied for one hour followed by incubation in secondary conjugates for 30 minutes. Cells were counterstained with 10mM Hoechst blue.
CHAPTER THREE

The role of both CD15 and CD15s in adhesion of NSCLC cells to brain endothelium
Cellular morphology of cell cultures

Phase contrast microscopy revealed morphological differences of cell lines according to culture and passage number. The monitoring of cell morphology and confluency also ensured that bacterial or fungal contamination in cell cultures would be detected. SC-1800 a human non-neoplastic astrocyte is an adherent cell culture, at passage 5 displayed slender cells with elongated processes, and a low number of dividing cells as seen in Figure 18A. hCMEC/D3 a human cerebral microvascular endothelial cell line, at passage 28 showed a homogenous spindle shape body and continuous monolayer without overlapping or random aggregation, as in Figure 18B. COR-L105, passage 25 is a human primary non-small cell lung cancer (NSCLC) cell line. It is an adherent culture with rapidly dividing cells, growing in a monolayer. Most cells appear to display nuclear atypia (Figure 18C). A549, passage 50 is a primary NSCLC cell line. A549 cells are round, adherent and rapidly dividing cells, growing in a continuous monolayer with a ‘honey-comb’ cell organisation (Figure 18D). UP-007 (passage 23) is a human high-grade glioblastoma multiforme cell line (GBM) cell line, derived from biopsy material cultured in-house. UP-007 culture shows high cell heterogeneity and this can be noted in mixed cell populations with some elongated cells with many processes at the end, some short more rounded cells and some big flat cells (Figure 18E). SNB-19 (passage 30) is a human established, homogenous high-grade glioblastoma multiforme cell line (GBM). Cells are short, small and lack the ability to form long processes (Figure 18F). SEBTA-001 (passage 4) is a human metastatic NSCLC established in-house from biopsy-derived materials from a patient with brain secondary tumour. SEBTA-001 showed a high level of morphological heterogeneity. Cells appear round with a tendency to grow in clusters or papillary structure (Figure 18G). SEBTA-005 (passage 3) is a human biopsy-derived metastatic NSCLC, established in-house from secondary tumour in the brain. Cells appeared large and homogenous monolayer. Cells tend to form multiple-processes. Cell-cell contact is rarely seen (Figure
112 H}. NCI-H1299 (passage 31) is an established cell line derived from a human metastatic lung carcinoma to cervical lymph node. Cells appeared to be rapidly dividing, round in shape and grow in a monolayer (Figure 18 I)

**Figure 18: Cellular morphology of studied cells.**

(A) SC-1800 a human non-neoplastic astrocyte cell line (passage 5), cells appear to be spindle shaped and stellate in culture. (B) hCMEC/D3 a human brain microvascular endothelial cell line (passage 28), reveals homogenous monolayer cell culture, spindle and polygonal cells with long and fine foot processes. (C) COR-L105 human primary lung NSCLC cells, passage 25 cells are large, round and multinuclear cells are occasionally seen. (D) A549 a human primary lung NSCLC cell line (passage 50), small, round cells with short, fine processes, mono-nuclear cells. (E) UP-007 a human grade IV GBM, passage 23, large cells with spindle or cylindrical shape. Cells have prominent nucleus. Multinuclear cells were occasionally seen. (F) SNB-19 a human grade IV GBM cell line (passage 30), small sized cells. (G and H) SEBTA-001(passage 4) and SEBTA-005 (passage3) respectively both are human metastatic NSCLC obtained from the brain passage. SEBTA-001 cells appear round and poorly differentiated while; SEBTA-005 cells are heterogeneous in nature. (I) NCI-H1299 a human metastatic lung NSCLC to cervical lymph node passage 31 cells appeared poorly differentiated. The images were obtained using a X10 objective via a phase contrast microscope. Scale bar= 20 μm.
Cell population-doubling time (PDT)

The population doubling time could then be defined from the exponential part of the growth by calculating the time taken for the cell to double (Table 8).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population doubling time (PDT) (hours)</th>
<th>Seeding density (cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-1800</td>
<td>71</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>24</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>COR-L105</td>
<td>21</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>A549</td>
<td>19</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>UP-007</td>
<td>23</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>SNB-19</td>
<td>20</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>SEBTA-001</td>
<td>28</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>SEBTA-005</td>
<td>31</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>NCI-H1299</td>
<td>18</td>
<td>$1 \times 10^7$</td>
</tr>
</tbody>
</table>

Table 8: Population doubling times and seeding densities of cells used throughout the project. Results are representative of three independent experiments carried out in triplicate.
3.1 Role of CD15 in adhesion of non-small cell lung cancer cells to brain endothelium

CD15 has been reported as a cell-cell adhesion molecule, it plays a key role in non-CNS metastasis and is overexpressed on various cancers. However, the role of CD15 in brain metastasis is largely unexplored. Intravasation and extravasation through the BBB are key steps for brain metastasis to occur; evidence points to the important role of endothelial cells in both lymphocyte trafficking and non-central nervous system (CNS) cancer cell metastasis (Strell S, Entschladen 2008). Although cancer cell extravasation has been intensively studied, the molecular mechanisms underlying metastasis to the brain are not fully understood. In particular, it is associated with human polymorphonuclear granulocytes and lung (Kadota et al., 1999), breast (Elola et al., 2007), prostate and kidney cancer cells. CD15 is crucial in the cell-cell recognition process (Streit and Stern 1995) and it was suggested that its absence from human glioma cells might explain why brain tumors rarely metastasize extraneurally. Recently CD15 has been identified as a potential cancer ‘stem-like’ cell marker in human and murine glioma spheroids (Mao et al., 1995 and Jelen et al., 2013). Significantly, it is a marker for metastatic lung adenocarcinoma (Kadota et al., 1999 and Nolte et al., 2013) being correlated with metastasis to non-CNS sites. However, the functional role of CD15 in metastasis from lung to brain remains obscure. CD62E is a cell-surface glycoprotein and CD15 ligand whose expression is induced by TNF-α, IL-1β and lipopolysaccharide (LPS) (Kaszubska et al., 1993). Currently, no definitive model of the metastatic process of cancer entry to the brain exists; previous studies in other tissues indicate that circulating cancer cell extravasation occurs in a similar manner to that of leukocytes with sequential steps of tethering, rolling, adhesion and transmigration studies on metastasis into colon and liver suggested interactions between selectins and their ligands regulate cancer cell adhesion (Brodt et al., 1997 and Tremblay et al., 2006)
*In vivo* study showed that, CD15 accumulates on the invasive edges of breast carcinoma boli, suggesting its potential role in metastasis (Brooks SA, Leathem 1995). Few studies have focused on the role of CD15 in metastasis to the brain. CD15 is rarely expressed on primary brain tumor cells; this observation providing a plausible explanation as to why glioma rarely metastasize extraneurally (Martin *et al*., 1995 and Pilkington *et al*., 1997) CD15 and E-selectin (CD62E) expression was demonstrated in both human primary and metastatic NSCLC cells using flow cytometry, immunofluorescence and Western blotting. The role of CD15 was investigated using an adhesion assay under static and physiological flow live-cell conditions. Human tissue sections were examined using. This study enhances understanding of cancer cell-brain endothelial adhesion and confirms that CD15 plays a crucial role in adhesion in concert with TNF-α activation of its binding partner CD62E.

This part of the project aims to provide a better understanding of CD15/CD62E interaction, enhanced by tumor necrosis factor-α (TNF-α), and its correlation with brain metastasis in non-small cell lung cancer (NSCLC).
3.1 CD15 expression in brain endothelial and NSCLC cell lines

CD15 cell surface expression and localization were characterized on brain endothelial cells, primary and metastatic NSCLC cells using ICC, Flow cytometry and WB analysis. Semi-quantitation of confocal images (Figure 19) demonstrated that when compared to isotype controls, CD15 immunoreactivity was highest on metastatic lung cancer cells NCI-H1299 (p<0.0001) followed by SEBTA-001 and SEBTA-005, A549, COR-L1299 and hCMEC/D3 respectively (Figure 19 A-B). There was no significant difference between CD15 expression in hCMEC/D3 compared to isotype control and to A549. There was a significant increase in CD15 expression compared to isotype control with positivity levels of NCI-H1299: 79%, SEBTA-001: 54%, SEBTA-005: 39%, COR-L105: 31%, A549: 23% and hCMEC/D3: 19.69% (Figure 19 C-D). There were no noted differences in CD15 expression in hCMEC/D3 compared with A549 and COR-L105. Western Blot results were consistent with these analyses (Figure 19 E).
Figure 19. Extracellular expression of CD15 in brain endothelial and lung cancer cell lines. A: Representative immunocytochemical images showing extracellular expression of CD15 in human brain endothelial cells (hCMEC/D3), human NSCLC metastatic cells obtained from cervical lymph node (NCI-H1290), brain (SEBTA-001 and SEBTA-005) and in non-metastatic NSCLC cells (A549 and COR-L105). B: Semi-quantitation analysis of CD15 cells from confocal images (A) using Zeiss’s ZEN image software. C: Representative flow cytometric histogram. D: Flow cytometric analysis of CD15 expression on hCMEC/D3, NCI-H1299, SEBTA-001, SEBTA-005, A549 and COR-L105. CD15 was highly expressed on NCI-H1299 and SEBTA-001 with less expression on COR-L105 and SEBTA-005 which expressed relatively the same amount. N=3, ***p<0.0001, **p<0.001 and *p<0.01. There was also less CD15 expression on A549 and hCMEC/D3 cells. E: Western blot of proteins from the cell lines showed highest CD15 expression in NCI-H1299, followed by SEBTA-001, SEBTA-005, COR-L105, A549 and hCMEC/D3. ABCE1 was used as a protein loading control.
3.2 TNF-α increases CD62E expression in human brain endothelial cells and NSCLC cell lines

MTS assay was used to determine the effect of TNF-α and TNF-β on the viability of hCMEC/D3 cells. Cells were incubated at different concentrations of TNF-α or TNF-β (0, 5, 10 and 25 pg/mL) and then cells viability was tested every 24 hours for 4 days. Results showed that TNF-α had no effect on hCMEC/D3 viability (Figure 20). However, TNF-β significantly reduced the cells viability (p<0.05) (Figure 20B), therefore TNF-α was selected for activating hCMEC/D3. TNF-α treatment of brain derived endothelial cells (hCMEC/D3), resulted in an increase in CD62E protein expression in a concentration dependent manner compared with non-stimulated cells (Figure 21A-C, E). To ensure this was a specific effect of TNF-α, CD62E expression was further examined in hCMEC/D3 cells treated with TNF-β (Figure 21A-B, D-E). ICC, flow cytometry and Western blotting were used to evaluate CD62E expression in brain endothelial cells cultured for 18 hours at three different concentrations of TNF-α and TNFβ: 5pg/mL, 10pg/mL and 25pg/mL. While CD62E expression was significantly higher in TNF-α treated hCMEC/D3 cells compared to the lung cancer cell lines (p<0.0001), there were also significant differences in CD62E expression within the group of lung cancer cell lines that were also treated for 18 hours with 25pg/ml of TNF-α (Figure 21 A-D). Semi-quantitation of confocal images (Figure 22A-B) demonstrated highest CD62E expression associated with hCMEC/D3 cells (Figure 22B). CD62E expression in hCMEC/D3 cells was significantly higher than CD62E expression in all the lung cancer cell lines tested (p<0.0001). CD62E expression in SEBTA-001cells was significantly higher than NCI-H1299 (p<0.01), SEBTA-005 (p<0.001) and A549 (p<0.0001). 73.88% of brain endothelial cells (hCMEC/D3) expressed CD62E and was significantly higher than isotype controls and the NSCLC cells (p<0.001). Flow cytometric analysis of lung cancer cells revealed CD62 positive cells in SEBTA-001.
(34.17%), in SEBTA-005 (27.6%), in NCI-H1299 (20.6%), in COR-L105 (32.7%), and in A549 (17.53%) (Figure 21 C-D).

Figure 20: TNF-α and TNF-β effect on hCMEC/D3 viability

Figure 20: TNF-α (A) and TNF-β (B) effect on human brain endothelium viability. Representative analysis of hCMEC/D3 cell viability following treatment with TNF-α and TNF-β, using MTS assay to assess cells viability. Results were obtained by N=3, *p< 0.05.
Figure 21. CD62E expression in TNF-α and TNF-β treated endothelial cells. 

A: hCMEC/D3 cells were positively stained for CD62E extracellular expression at different TNF-α and TNF-β concentrations (green). B: Western blotting of hCMEC/D3 cultured in increasing concentrations of TNF-α and TNF-β. C, D: Overlay histogram of flow cytometric analysis of CD62E expression in hCMEC/D3 cells cultured with different concentration of TNF-α and TNF-β. E: Flow cytometric analysis of CD62E expression in hCMEC/D3. N=3, ***p<0.0001, **p<0.001, *p<0.01.
Figure 22. CD62E expression in TNF-α treated NSCLC cell lines. 

A: Representative immunocytochemical images showing expression of CD62E in cancer cell lines following treatment with TNFα (25pg/ml). CD62E was highly expressed and well-distributed across cell membrane of hCMEC/D3 cells, at lower levels on NSCLC metastatic cells (NCI-H1299 SEBTA-001 and SEBTA-005) and primary NSCLC cells (A549 and COR-L105). 

B: Semi-quantitation analysis of CD62E cells from confocal images (A) using Zeiss’s ZEN image software. 

C: Overlay histogram of flow cytometric analysis of CD62E expression in cells treated with TNFα (25pg/ml). 

D: Flow cytometric analysis of CD62E expression in NCI-H1299, SEBTA-001, SEBTA-005, A549 and COR-L105. N=3, ***p<0.0001, **p<0.001, *p<0.01.
3.3 CD15 and CD62E mediate adhesion of NSCLC to hCMEC/D3 monolayer under static conditions

We first wanted to determine if the TNF-α increased CD62E in endothelial cells led to an increase in tumor cell adhesion. hCMEC/D3 cells were plated and treated with TNF-α or TNF-β for 18 hours, followed by several washes and addition of serum free media containing lung cancer cells pre-labeled with a green fluorescent dye (Cyto Tracker™). Following 90 minute incubation, co-cultures were washed and cells were lysed and evaluated for levels of fluorescence. hCMEC/D3 cells incubated with TNF-α resulted in a significant increase in NSCLC adhesion with the highest relative fluorescent units associated with the NCI-H1299 cells followed by the lung cancer cells (Figure 23A, p<0.001). There was no effect on adhesion when hCMEC/D3 cells were treated with TNF-β (Figure 23A). To rule out that a change in viability via TNF-α may have contributed to this increase in adhesion, viability assays were conducted. There was no change in hCMEC/D3 cell viability following TNF-α treatment (Figure 24). However, hCMEC/D3 cells treated with TNF-β demonstrated significantly less viability when measured over four days (Figure 24). The time course for the adhesion assay described above consisted of an 18 hr incubation with TNF-α or TNF-β and then the cancer cell lines were added and allowed to adhere for 90 minutes. Therefore the change in cell viability seen with TNF-β at later time points would not have accounted for the lack of adhesion (Figure 23A). In a separate method to evaluate adhesion, we used confocal image analysis. The same conditions used in the assay above were repeated except on coverslips and prepared for ICC. Semi-quantification of confocal images using Zeiss Zen software revealed that the metastatic cell lines NCI-H1299 cells were the most adherent, followed by metastatic cells (SEBTA-001 and SEBTA-005) and primary NSCLC cells (COR-L105 and A549) (Figure 23B). These results suggest a strong correlation between CD15 expression and NSCLC cell adhesion. To determine whether CD15 plays a key role in NSCLC cell adhesion to
brain endothelial, an adhesion assay was conducted in the presence of CD15 antibodies or isotype (IgM) controls (Figure 23C). CD15 mAb-blocking significantly decreased metastatic (NCI-H1299, SEBTA-001 and SEBTA-005) and primary NSCLC (COR-L105 and A549) adhesion compared to non-treated cells and cancer cells adhere on hCMEC/D3 endothelial culture while, after CD15 blocking cells sit on the surface but are not attached. Furthermore, there was no significant change in adhesion of cancer cells due to blocking with non-specific Isotype (IgM) (p<0.0001) (Figure 23 C). In addition, there were no observed toxic effects of antibody incubation with the various cancer cell lines or the endothelial cell line (Figure 24).
Figure 23. NSCLC adhesion to TNF-α treated brain endothelial cells and the effect CD15 immunoblocking. 

A: Qualitative adhesion of NSCLC cells on human brain endothelial cell monolayer. Primary and metastatic NSCLC cancer cells were incubated for 90 minutes on a monolayer of activated hCMEC/D3 cells. Non-adherent cancer cells were washed away and adherent cells were lysed and quantified via a microplate reader at 480-520nm. N=3, P<0.0001=***. The results showed the strong effect of CD62E, once stimulated by TNF-α on lung tumour cells (red bars). Absence of TNF-α showed a significant decrease in cancer cells adhesion (white bars) (P<0.001=***).

B: Confocal Images (Top panel) showing adhesion of green fluorescently labelled NSCLC cells on brain endothelial cell monolayer (blue) and semi-quantitation analysis of confocal images (lower panel) using Zeiss’s ZEN image showing a significant increase in NCI-H1299, SEBTA-001, and SEBTA-005 adhesion to TNF-α treated hCMEC/D3 cells. N=3, ***p<0.0001, **p<0.001, *p<0.01.
Figure 23C: Quantitative adhesion of human primary and metastatic NSCLC cells blocked with CD15 mAb (red bar) and with non-specific Isotype IgM (white bar) to assess CD15 mAb-blocking efficiency and specificity. Adhesion of NSCLC cells on a monolayer of hCMEC/D3 (grey bar) act as a negative control. N=3, P<0.0001=***.

Figure 24: CD15 mAb blocking effect on cell viability

Figure 24. CD15 mAb blocking effect on cell viability. Effect of CD15 mAb-blocking on hCMEC/D3 and NSCLC cell viability. There were no significant difference in cell viability in cells treated with CD15 mAb-blocking compared to non-treated cells. (N=3), p>0.05=ns.
3.4 CD15 mAb-blocking decreases adhesion of NSCLC cells under shear stress

To determine if the adhesion results obtained from static experiments would hold when experimental conditions were conducted under shear stress, we used the Vena 8 Endothelial+ biochip and micro-pump (Cellix-Ireland) and conducted live cell microscopy to determine the effect of CD15 immunoblocking on dynamic adhesion of metastatic lung to brain cancer cells (SEBTA-001) over a 40 minute time range with a perfusion rate at 2.5 dyn/cm² of fresh medium (Figure. 25A). CD15 mAb-blocking was shown to decrease the number of adherent cancer cells on the brain endothelial cell monolayer lining the Vena 8 biochip channel compared to non-blocked cells (Figure. 25A, lower panel and side graph). The aggregation of the SEBTA-001 cells seen in Figure 25A (arrows) without immunoblocking suggests homophilic binding of CD15 on cancer cells as well as heterophilic binding between CD15 and CD62E. This is supported by confocal images of ICC performed on co-cultures of green fluorescently tagged metastatic cancer cells (SEBTA-001) adhering on a monolayer of activated brain endothelial cells (Figure. 25B-C). Results showed prominent and condensed expression of CD15 on adherent cancer cell surface and cell processes (Figure. 25C arrows). Both CD15 and CD62E were also localized at the site of cancer cell-brain endothelial cell adhesion and CD62E was seen distributed on the activated endothelial cells and co-localised with CD15 supporting heterophilic adhesion sites between cancer cells and brain endothelial cells (Figure. 5C arrows). To confirm CD15 and CD62E localisation at the site of adhesion, three-dimensional confocal images created from z-stacks (Figure. 5D) was analysed in culturing SEBTA-001 on a monolayer of endothelial cells.
Figure 25. CD15 mAb-blocking reduces adhesion of NSCLC cells under dynamic conditions.

A (upper panel)

![Image showing a dynamic cell adhesion assay](image)

**Without CD15 mAb-blocking**

10 mins | 20 mins | 30 mins | 40 mins
---|---|---|---

**CD15 mAb-blocking**

10 mins | 20 mins | 30 mins | 40 mins
---|---|---|---

A (lower panel)

![Image showing relative fluorescent units](image)

**CD15 mAb-blocking**

**No CD15 mAb-blocking**

Relative fluorescent units vs. Exposure time (minute)

Figure 25. CD15 mAb-blocking reduces adhesion of NSCLC cells under dynamic conditions. **A:** A dynamic cell adhesion assay was carried out on highly brain metastatic cells (SEBTA-001) using an AxioVert 200M microscope (C.Zeiss, UK) within an environmentally-controlled incubator. SEBTA-001 cells were incubated with isotype control (IgM) or CD15 mAb followed by perfusion of 1x10⁶ cells over a monolayer of hCMEC/D3 cells at 2.5 dyn/cm² for 40 minutes. Phase contrast and fluorescent images were acquired at real-time every 10mins with a X5 objective using Volocity software. Scale bar=20μm. **Right panel:** Representation of (left panel) in relative fluorescent units of SEBTA-001 cell adhesion with and without CD15 mAb for 10, 20, 30 and 40 minute time points.
Figure 25. **B-D:** Confocal images of green-fluorescently labelled adherent brain to lung metastatic cancer (SEBT-001) cells cultured on a monolayer of hCMEC/D3 cells (blue). **B:** CD15 expression (red) on the edges of SEBT-001 (green). ICC images showed expression of CD15 (red) on the adherent cancer cells (green) on a monolayer of human brain endothelial cells stained with Hoechst blue **C:** The merged image showed expression of CD15 (red) on adherent tumour cells (SEBTA-001) (green) on an activated monolayer of human brain endothelial cells (blue) expressing CD62E (purple). **D:** Optical sections of three-dimensional confocal image created from z-stack. The top and lower view represents one image/different angles through the z-stack showing an adherent SEBTA-001 expressing CD15 (purple) on an activated monolayer of brain endothelial cells expressing CD62E (red). Right side view represents an optical section through the depicted z-stack shows the precise interaction between CD15 and CD62E during NSCLC-brain endothelium cell adhesion.
3.5 CD15 and CD62E expression in human biopsy of lung to brain metastasis

Immunohistochemistry on paraffin embedded formalin fixed tissue sections of human lung metastasis to brain biopsies using antibodies to both CD15 and CD62E was conducted (Supplemental figure 3). Both CD15 and CD62E positive cells could be seen throughout the tumor core whereas in adult normal brain cortex neither was detected. In patient 1 there was an area of host tumor interface where CD15 positive cells were detected within vessels, and in the same patient CD62E was associated with endothelial cells lining the vessels.
Figure 26: CD15 and CD62E immunohistochemistry in human derived biopsy tissue.

Representative images from 3 patient derived lung to brain metastasis, 1 control human brain and 1 human Hodgkin lymphoma (Reed-Sternberg cells) as a positive control for CD15 staining. Both CD15 and CD62E expression was detected in tumor core in all metastatic cases examined (n=5). In one case (patient 1) an area of host tumor interface was examined as seen in the top panel. CD15 positive cells were detected in host brain tissue and associated with blood vessels and CD62E staining associated with the lining of capillaries. Biopsies from metastatic brain lesions were obtained from frontal lobe (patient 1-2) and parietal lobe (patient 3). Images were obtained using an Ariol microscope (Leica, Germany) at x40 magnification.
3.6 CD15s-CD62E interaction mediates the adhesion of non-small cell lung cancer cells on brain endothelial cells during metastasis to the brain.

CD15s (SL$	ext{e}^3$) is a tetra-polysaccharide cell-cell adhesion molecule, it acts as a ligand for E-selectin and P-selectin (Polley et al., 1991) and involved in adhesion of cancer cells to endothelial cells (Vestweber and Blanks 1998, Giavazzi et al., 1993, Kitayama et al., 2000; Burdick et al., 2003). It mediates the initial steps of tethering and rolling of white blood cells during WBC homing process. The circulating cells head to the site of injury by initial binding between CD15s and E-selectin (Munro 1993; Aruffo and Glycosci 1994). Burdick et al., (2003) highlighted that metastatic colon adenocarcinoma cells adhere to human umbilical vein endothelial cells (HUVECs), through CD15s-glycolipids and CD15s-glycoproteins which bind to E-selectin. Wang et al., (2003) reported that CD15s expression is associated with malignancy of human non-small pulmonary cancer (NSCPC) and human primary liver cancer (PLC) tissue while they are not expressed in the adjacent non-malignant tissue. Moreover, Ikeda et al.,(1996) showed that CD15s is expressed in 60% of patients with primary lesions of gastric cancer and in 51% of patients with advanced primary gastric tumour and metastatic lymph nodes. It is further reported that expression of CD15s may be used as a marker for the early diagnosis of cervical cancer (Nakagoe et al., 2002). Very little is however, known about CD15s in the CNS but we have recently reported that overexpression of CD15s is seen to be correlated with cell arrest in G1 phase of cell cycle in glioblastoma cells (GBM) and metastatic lung to brain cancer cells (Jassam et al., 2015b). This part of the project indicates the important role of CD15s/E-selectin heterophilic interaction, in cancer cell adhesion. Also, the study reveals that CD15s may play a crucial role in homing process of NSCLC cells during brain metastasis.
3.7 Characterisation of CD15s in brain endothelium and NSCLC cultured cells

Localisation and extracellular expression of CD15s were characterised in human brain endothelial cells as well as primary and metastatic NSCLC cells via ICC, WB and flow cytometry analysis. CD15s was localised and prominently expressed on the surface of brain metastatic lung cancer cells (SEBTA-001 and NCI-H1299). Lower levels of expression were seen on the surface of brain endothelial cells and on primary lung cancer cells (COR-L105 and A549) compared with the isotype control (Figure 27A). Semi-qualitative Western blotting analysis showed intense immunopositive bands in the metastatic cells while less intensity was observed in primary cancer cells and endothelial cell membrane extracts (Figure 27B). Qualitative analysis of flow cytometry showed highest levels of CD15s positivity were observed in metastatic lung cancer cells: NCI-H1299 (39% positive) and SEBTA-001 (35% positive). Lower level was seen in brain endothelial cells (25% positive) followed by the primary lung cancer cells: COR-L105 (21% positive) and A549 (18% positive), (Figure 27C and D).
Figure 27: Expression of CD15s in brain endothelial and NSCLC cells

A: Representative ICC images showing CD15s in hCMEC/D3, NCI-H1299, SEBTA-001, A549 and COR-L105
B: Western blot analysis of CD15s of proteins from the cell lines showed highest CD15s expression in NCI-H1299 and SEBTA-001 followed by hCMEC/D3, COR-L105 and A549. ABCE1 was used as a protein loading control.
C: Representative flow cytometric histogram and D: Quantitative flow cytometric analysis of CD15s expression on hCMEC/D3, NCI-H1299, SEBTA-001, COR-L105 and A549. CD15s was overexpressed in NCI-H1299 and SEBTA-001 with less expression in hCMEC/D3, COR-L105 and A549. N=3, ***P<0.001, **P<0.01 and *P<0.05.
3.8 Interrupting CD15s-CD62E interaction reduces the adhesion of cancer cell-brain endothelium

To explore the role of CD62E in adhesion of NSCLC cell-brain endothelium we conducted a qualitative and quantitative adhesion assays under static conditions using a confocal microscopy and microplate reader to assess the relative number of adherent NSCLC cells. CD62E expression was activated by TNF-α (25pg/mL) and then green fluorescently tagged NSCLC were applied onto activated and non-activated brain endothelia cells. Findings showed that absence of CD62E (non-activated cells) significantly reduced the adhesion of all cancer cells (P<0.001), (Figure 28A and B) compared with the high numbers of adherent cells on the activated brain endothelial cells that expressing CD62E. These results suggested that CD62E and TNF-α have a key role in adhesion of NSCLC during seeding into the brain.
Figure 28: Role of CD62E in adhesion of NSCLC cells-brain endothelium

Figure 28. Role of CD62E in adhesion of NSCLC cells-brain endothelium. 

A: Qualitative adhesion of NSCLC cells onto brain endothelium monolayer were grown on glass coverslips, green fluorescent NSCLC cells were applied onto a monolayer of hCMEC/D3 cells and incubated for 90 minutes with and without activation via TNF-α, non-adherent cells were washed out and co-cultures were fixed and examined by confocal microscope. 

B: A Quantitative adhesion of NSCLC cells were conducted to verify the quantitative results, hCMEC/D3 cells were seeded into 96-well plate, then green fluorescent tagged NSCLC cells were applied on the hCMEC/D3 monolayer, after 90 minutes incubation the non-adherent cells were washed out and adherent cells were lysed followed by quantification via microplate reader at 480-520nm. Results showed a strong reduction caused by absence of TNF-α (White bar) compared to TNF-α stimuli. N=3, ***P<0.001, **P<0.01 and *P<0.05. Scale bar=20µm
3.9 Immunoblocking of CD15s reduced the adhesion of cancer cell-brain endothelium under static conditions

A qualitative adhesion assay under static conditions was performed using a confocal microscopy and a quantitative assay via a plate reader to assess the role of CD15s in adhesion. Results showed that metastatic cancer cells (NCI-H1299 and SEBTA-001) were more adherent than the primary lung cancer cells (COR-L105 and A549) (Figure 29 A and B) and immunoblocking of CD15s was significantly (P<0.001) reduced the relative number of adherent cancer cells onto an activated brain endothelial cell monolayer. These results suggested a correlation between the expression of CD15s and adhesion of lung cancer cells (Figure 29 A). In addition, mAb-immunoblocking against CD15s reduced the adhesion ability of cancer cells compared to the adhesion ability of cancer cells without mAb-CD15s immunoblocking. However, no reduction in adhesion was detected during blocking with non-specific isotype (IgM) monoclonal antibodies; these results assessed the specificity of mAb-CD15s blocking and validated the correlation of CD15s and adhesion ability of cancer cells under static conditions (Figure 29B).
Figure 29 A: CD15s immunoblocking reduced the adhesion of lung cancer cells under static conditions. Confocal images (top panel) showing adhesion of green fluorescently labelled NSCLCs on a brain endothelial cell monolayer (blue) and Semi-quantitative analysis of confocal images (lower panel) show a significant decrease in adhesion ability of NSCLC cells to adhere to hCMEC/D3 cells. N = 3, ***P<0.001, **P<0.01, *P<0.05. Scale bar=20µm. In a previous study the optimal concentration of TNF-α was optimised (Jassam et al., 2015) the same concentration of TNF-α was used to activate the expression of CD62E on brain endothelial cells. Brain endothelial cells (hCMEC/D3) were exposed to 25 pg/mL of TNF-α for 18 hours and levels of CD62E were detected using flow cytometry (Data not shown).
Figure 29 B: Blocking with CD15s mAb significantly reduced the adhesion of NSCLC to brain endothelium.

Figure 29. **B:** Blocking with CD15s mAb significantly reduced the adhesion of NSCLC to brain endothelium. Quantitative adhesion assay of human primary and metastatic lung cancer (NSCLC) cells blocked with CD15s mAb (blue bar), non-specific isotype IgM (white bar) to assess the efficiency and specificity of CD15s mAb-blocking efficiency and NSCLC cells on a monolayer of hCMEC/D3 (grey bar) as a negative control. N= 3, ***P<0.001.
3.10 CD15s mAb blocking decreases adhesion of NSCLC cells under shear stress condition

To examine whether CD15s plays a role in adhesion of cancer cells under physiological shear stress (blood flow) as well as its role under static conditions, an activated endothelial monolayer was allowed to grow on a Vena8 endothelial+ biochip and green fluorescent tagged NSCLC cells were perfused onto the endothelial monolayer via a micropump (Cellix). Live cell microscopy was then conducted to monitor the effect of CD15s immunoblocking on the adhesion cancer cells. Highly metastatic lung to brain cancer cell line (SEBTA-001) was perfused at a rate of 2.5 dyn/cm² with warmed fresh EBM+EGM2 medium supplemented with 2% of human serum and 25pg/mL TNF-α.

Cells adhesion was then studied over a 40-minute time range. Results showed that SEBTA-001 cells started to adhere onto activated endothelial monolayer where no CD15s immunoblocking and the number of adherent cells was increased in a time dependent manner (Figure 30). In parallel, same number of SEBTA-001 cells (previously incubated with mAb-CD15s for 10 minutes) were perfused and this time with mAb-CD15s the cancer cells were seen to stay in a suspension rather than adhering on the endothelial cells as observed in the non-blocked well (Figure 30). These findings confirmed the key role of CD15s in adhesion of lung cancer cells on the brain endothelial cells under physiological shear stress conditions.
Figure 30. Immunoblocking with CD15s mAb significantly decreased the adhesion ability of NSCLC under dynamic conditions (2.5 dyn/cm²).

Figure 30. Immunoblocking with CD15s mAb significantly decreased the adhesion ability of NSCLC under dynamic conditions (2.5 dyn/cm²). A dynamic cell adhesion assay was carried out on highly metastatic brain cells (SEBTA-001) using an AxioVert 200 M microscope (C. Zeiss). **Top panel:** cancer cells were incubated with isotype control (IgM) or CD15 mAb, followed by perfusion of 1×10⁶ cancer cells over a monolayer of hCMEC/D3 cells at 2.5 dyn/cm² for 40 minutes. Phase contrast and fluorescent images were acquired at real time every 10 minutes with an X5 objective using Volocity software. Scale bar= 20 μm. **Lower panel:** results in relative fluorescent units of adherent cancer cells with and without CD15 mAb for 10, 20, 30, and 40 minute time points are given in the lower graph.
3.11 CD15s expression was localised at the adhesion sites of cancer cell-brain endothelium

Finally, we conducted a microscopic study on a co-culture of adherent cancer cell (SEBTA-001) onto a monolayer of brain endothelial cells. The cancer cells were left to adhere and the co-culture was fixed before immunocytochemistry was performed against CD15s and CD62E. Results showed a prominent expression of CD15s was seen on the surface of green fluorescently tagged cancer cells (red) with accumulation on the surrounding outer edges and processes of the adherent cancer cells (Figure 31 A). Also, CD15s and CD62E were both co-localised at the site of NSCLC cell adhesion where CD15s (red) was seen to be expressed on green-tagged cancer cells and CD62E (purple) was observed on the surface of the activated brain endothelial cells at the site of the adhesion (Figure 31 B). These images assessed the involvement of CD15s-CD62E interaction in adhesion process of NSCLC cells onto brain endothelium.
Figure 31. Localisation of CD15s on the surface of adherent SEBTA-001 cells at the site of adhesion. Confocal image of green fluorescently-labelled adherent brain to lung metastatic cancer cells (SEBTA-001) on a monolayer of activated hCMEC/D3 cells (blue). **A:** Expression of CD15s (red) (white arrows) was seen on the edges of adherent cells SEBTA-001 (green) at the site of adhesion on brain endothelial cells. **B:** Expression of CD15s (red) (white arrows) was observed on surface of adherent cancer cells SEBTA-001 (green) and CD62E (purple) (white arrows) was seen on the monolayer of activated brain endothelial cells (hCMEC/D3) at adhesion site of cancer cell-brain endothelium. Scale bar=20μm.
3.12 CD15s expression in human biopsy of lung to brain metastasis

We showed that in vitro that CD15s and CD62E are expressed on the NSCLC and brain endothelium respectively. In addition, we showed their functional role in adhesion of NSCLC cell-brain endothelium in vitro. Then we wanted to explore if it is the same case in vivo; thus, paraffin embedded of formalin fixed tissue sections of human lung to brain metastatic tumour biopsy and healthy brain tissue section were immune-stained against CD15s and CD62E. Immunohistochemistry results showed that CD15s was expressed in brain metastatic tumour section, CD15s positive cells were seen throughout the tumour core and in the distant local lesions also, there was an area of host tumour interface where CD15s positive cells were detected within vessels. Whereas, no CD15s expression was noted in the non-malignant adjacent host brain tissue, as well as, CD15s was not seen in human healthy normal brain tissue section. In the same biopsy CD62E was observed to be expressed on the inner lining endothelial cells of the brain microvessels, however no expression were detected in the healthy normal brain section. Again, these in vivo results pointed to the importance of CD15s and CD62E in brain metastasis (Figure 32).
Figure 32: CD15s and CD62E immunohistochemistry in human derived biopsy tissue.

Representative Immunohistochemistry images from one patient with lung-brain metastasis. CD15s was detected in tumour core and infiltrated into non-neoplastic host brain tissue. Images were obtained using an Ariol microscope (Leica) at x20 and x40 magnification. Scale bar=20µm.
3.13 Discussion

3.13.1 Role of CD15-CD62E interaction in adhesion of NSCLC-brain endothelium

Three quarters of brain tumors are metastatic cancers originating from primaries in distant organs; the brain is known to be key target of secondary NSCLC, 20–40% of lung cancer patients develop oligo- or multiple- brain metastasis during their illness (Ali et al., 2013). Metastasis to the brain is a complicated multi-step process requiring interaction between metastatic cancer cells and target environment. However, these interactions are not yet fully understood, particularly the adhesion of cancer cell on brain endothelium in early stages of entry into the brain. CD15 is cell adhesion fucosylated carbohydrate, expressed on leucocytes and various types of non-CNS cancer cells (Gout et al., 2006) but rarely expressed in human brain (Pilkington et al., 1998). Moreover, CD15 overexpression has been correlated with a progression to metastatic stage. Indeed, significant correlation exists between CD15 overexpression and colorectal cancer metastasis, through prominent CD15 expression on the invading edges of cancer lesions (Gout et al., 2006). CD15 is involved in the extravasation process through its interaction with selectins particularly E-selectin (CD62E), a glycosylated transmembrane and cellular adhesion molecule crucial for homing of circulating cells through its expression on endothelial cells (Gout et al., 2006) CD62E facilitates cancer cell adhesion to endothelial cells in various cancers such as, colon (Gout et al., 2006), breast (Lafrenie et al., 1994) and lung (Martin-Satue et al., 1998) Here we hypothesized that CD15 and CD62E interaction is involved in cancer cell adhesion during metastasis to the brain. Previous studies have shown that human bone marrow microvascular endothelial cells play an important role in metastasis to the brain via interaction between CD62E on endothelial cells and its ligands on metastatic neoplasms (Fazakas et al., 2011). However, most studies have used non-specialized experimental conditions such as human cells cultured in fetal calf serum, using non-specific medium and using non-brain derived endothelium. In our study, we used 2% human serum and medium
supplemented with specific growth factors for each cell line to maintain cell differentiation and characteristics. We used human brain endothelial cells (hCMEC/D3) established from temporal lobe microvessels from an epileptic patient then immortalised by introducing human telomerase or SV40 T antigen employing lentiviral vectors (Kiem et al., 2009). Previously, HUVECs have been used to study metastasis to the brain (Martin-Satue et al., 1998) however; functional differences were demonstrated between leukocyte adhesion on HUVECs and on brain derived endothelium (Rizzo et al., 2014 and Man et al., 2008). In the present study, CD15 was overexpressed on metastatic NSCLC cell membranes (NCI-H1299, SEBTA-001 and SEBTA-005); while there was a lower expression on primary NSCLC cells (COR-L105 and A549). Moreover, CD15 was characterised on SEBTA-001 and SEBTA-005 (established in house metastatic NSCLC cell lines obtained from brain). CD62E, the natural ligand for CD15 was upregulated on brain endothelial cells (hCMEC/D3) and cancer cells in response to a TNF-α inflammatory stimulus, Also CD62E was expressed on the lung cancer cells, which is expected as they are carcinoma cells (epithelial origin); and that may suggest CD62E as a possible therapeutic target. Both mAb-blocking of CD15 and absence of CD62E/TNF-α correlated with significantly decreased adhesion of cancer cells on brain endothelium. Confocal microscopy revealed the expression of CD15 around adherent cancer cells and to localize CD15/CD62E interaction at adhesion sites of cancer cells/brain endothelial cells; these findings suggests that CD15 and CD62E play important roles in adhesion of NSCLC cells to brain endothelium in static conditions. We then explored the possible effects of vascular blood flow by combining live cell imaging and shear stress fluidics twinned with CD15 mAb-blocking under TNF-α immune stimulus using in vitro model developed from human cell cultures maintained in a specialized environment to mimic in vivo environment inside blood brain microvessels. For this a microfluidic chip (Vena8-Cellix) was used with low shear stress 2.5 dyn/cm² and volumetric Flow Rates 10mL/hr mimicking the low flow in brain microvessels (Mintun et al., 2001). Under these conditions, adhesion of cancer cells
was significantly decreased by absence of CD15 or CD62E/TNF-α, suggesting the critical role of CD15 and CD62E/TNF-α in cancer cell adhesion during early stages of cancer cell extravasation. While in vivo data are lacking for NSCLC, a recent study reinforced the importance of CD62E in breast to brain metastasis in mice, by showing that the adhesion of breast cancer cells to brain endothelial cells was enhanced by the presence of VCAM-1/VLA-4, ALCAM-1 and (Soto et al., 2014). Our in vitro study has also shed light on the functional characterization and localization of CD15 and CD62E on the site of NSCLC seeding to the brain. CD15 expression levels correlated with the adhesion of cancer cells to stimulated brain microvascular endothelial cells. NSCLC metastatic cells obtained from brain lesions (SEBTA-001 and SEBTA-005) were more adhesive than the primary NSCLC cells (COR-L105 and A549). These results are consistent with previous studies that referred to the correlation between elevated level of CD15 and metastasis in different types of non-CNS cancer (Nolte et al., 2013 and Fazakas et al., 2011). CD15 mAb-blocking resulted in a decrease in the number of adherent cells on stimulated brain microvascular endothelium (hCMEC/D3) under both static and dynamic conditions. Thus, this study indicates CD15 as a possible target for prevention of brain metastasis in NSCLC patients. While this in vitro experimental model adds to our knowledge of metastatic cell adherence to brain endothelium, there are limitations in that it does not include other cell types that are known to be present. For example, the influence of circulating neutrophils in brain metastases and their role in this process is not known and is currently under investigation. Although little is known concerning the histopathology of CD15 expression in CNS tumours (Reifenberger et al., 1992) and human lung cancer to brain metastasis, there have been reports of CD15 expression in NSCLC and more recently associations with lung cancer stem-like cells. A more rigorous characterization of CD15 in human lung cancer to brain derived biopsy is now in progress.
3.13.2 Role of CD15s-CD62E interaction in adhesion of NSCLC-brain endothelium

Brain metastasis is a complicated, multi-step process, despite its great clinical importance, the underlining molecular mechanisms are still not fully understood. Heretofore, the highest rate of brain metastases are originated from lung primaries and 20-40% of non-small cell lung cancer (NSCLC) patients develop a brain metastasis at or within a short period of primary tumour diagnosis (Ali et al., 2013). Metastasis to the brain requires a well communication between the metastatic cells and its microenvironment, circulating cancer cells have to go through tethering, rolling, adhesion and transmigration during seeding into the brain. Although, not much known about the molecular bases of these interactions particularly, the adhesion process of cancer cell-brain endothelium. CD15s is a tetrasaccharide cell-cell adhesion and cell recognition molecule (Haselhorst et al., 2001), it is involved in adhesion of lymphocyte during homing process (Turunen et al., 1994). CD15s overexpression was reported to be correlated with distance metastasis in lung patients (Fukuoka et al., 1998). As well as in colorectal metastatic cancer (Nakamori et al., 1997) and liver metastasis where, CD15s inaction with selectins mediate adhesion of cancer cells onto liver endothelium (Izumi et al., 1995). In addition, elevated levels of CD15s were revealed to be associated with metastasis in head and neck cancer (Renkonen et al., 1999). Our study showed that CD15s was expressed on cell surface of brain endothelial cells (hCMEC/D3), primary NSCLC cells (A549 and COR-L105) and the highest levels of expression was observed on the metastatic NSCLC obtained from cervical lymphnode (NCI-H1299) and metastatic NSCLC obtained from the brain (SEBTA-001) (Figure 1), these results agreed with Fukuoka et al., 1998 findings who, revealed that CD15s high expression was correlated with the metastasis of lung carcinoma. Same findings were shown by Kadota et al., 1999 suggesting CD15s correlation with distant metastatic lesions in lung carcinoma. Recently, elevated level of CD15s was revealed to be used as an independent marker to predict NSCLC patients at the metastasis stage (Komatsu
et al., 2013). Although, not much known about CD15s expression in CNS malignancies. However, we have shown in a previous study that CD15s overexpression was cell cycle dependent in high grade glioblastoma cells as well as in metastatic brain tumour cells originated from lung (Jassam et al., 2015b). It has been shown that brain endothelial cells play a key role in metastasis to the brain (Soto et al., 2014). In our study, we have used an adult human brain microvascular endothelial cell line (hCMEC/D3) established from microvessels of temporal lobe obtained from epileptic patient, these cells were successfully immortalised by transfection with lentiviral vector with human telomerase (SV40T antigen) (Hatherell et al., 2010), hCMEC/D3 was used as a BBB in vitro model; in previous study we have optimised the extracellular expression of CD26E which was enhanced by 25pg/mL of TNF-α (Jassam et al., 2015b). hCMEC/D3 cells were grown and maintained in highly specialised medium supplemented with all essential factors and cytokines to maintain hCMEC/D3 original cellular characteristics in addition to, all the studied cells were maintained in medium supplemented with human serum to avoid any possible functional changes enhanced by bovine serum (Murray 2010). However, non-CNS endothelial cells such as HUVECs were used to study metastasis to the brain (Martin-Satue et al., 1998), Also, a functional differences were noted in adhesion of leukocytes on brain endothelial cells and adhesion on umbilical vein endothelial cells (Man et al., 2008). The importance of CD15s in cell-cell adhesion is due to its ability to interact with endothelial adhesion molecule as E-selectin (CD62E), as in lymphocytes homing process (Munro et al., 1992). Interestingly, our findings showed a correlation between expression of CD15s and adhesion ability, less adherent cells were noted in primary lung cancer cells (COR-L105 and A549) compared to a high number in metastatic cells (NCI-H1299, SEBTA-01 and SEBTA-005). A significant reduction in NSCLC adherent cells was caused by the absence of CD62E where, adhesion assay was conducted without TNF-α stimuli; Earlier study has shown the involvement of CD15s-CD62E interaction in metastasis in different non-CNS and revealed that entrapping the interaction between CD15s and CD62E largely
reduced the adhesion of lung carcinoma, colon and macrophages (Zeisig *et al*., 2004). We found a significant reduction (p<0.01) in adhesion of lung cancer cells was caused by blocking with monoclonal antibodies against CD15s. These results suggest that both CD15s and CD62E play an important role in adhesion of cancer cell onto brain endothelium under static conditions. We then moved to study whether blocking of CD15s reduces the adhesion of cancer cells under flow. Thus, we conducted live cell imaging of cell-cell adhesion under shear stress at perfusion rate 2.5 dyn/cm² and volumetric flow rate 10mL/hr resembling the blood flow in human brain microvascular system (Mintun *et al*., 2001). Using highly metastatic NSCLC cells obtained from brain (SEBTA-001), results showed that blocking with antibodies against CD15s (reduced cells adhesion compared with non-blocked cells. Microscopic study showed a localisation of CD15s surrounding the adherent cells and co-localisation of CD15s/CD62E was seen at the site of adhesion of metastatic lung cancer cells (SEBTA-001) onto brain endothelial cells, these findings suggesting CD15s and CD62E play a key role in lung cancer cell adhesion. In addition, CD15s were seen to be expressed in human tissue section of metastatic lung to brain tumour. While, no CD15s was detected in the healthy brain tissue section. Although, not much known about in vivo and *in vitro* characterisation of CD15s expression in human CNS however, a single study revealed that CD15s was not expressed in any type of normal brain cells apart from microglia (Satoh and Kim, 1994). The recent study showed a highly specialised *in vitro* experimental model to study cell-cell adhesion of cancer cells during lung-brain metastasis and added new findings regarding CD15s expression in human brain.
CHAPTER FOUR

The role of CD15 and CD15s on transendothelial migration of NSCLC cells
4.1 The role of CD15 and CD15s on transendothelial migration of NSCLC cancer cells.

The highest incidence of brain metastasis is seen in lung cancer patients (40-50%) followed by breast (20-30%) and melanoma (5-10%) (Wen and Loeffler, 2000). Metastasis to the brain is considered to be the cause of 95% of cancer related deaths (Denkins 2004 and Sloan 2009). The BBB is a selective defensive barrier which maintains a highly specific environment within the CNS by inhibiting both fluctuations of plasma components as well as providing entry of substances that may potentially cause brain toxicity (Abbott et al., 2006 and Hatherell et al., 2011). The BBB is composed of endothelial cells, a basement membrane, pericytes and endfeet of astrocytes which form the neurovascular unit (Abbott et al., 2006). Endothelial cells of the BBB are specific to the BBB and on low pinocytosis and no fenestrations (Kis et al., 2006). It has been reported that endothelial cells have a crucial role in the homing process of brain metastasis from breast cancer (Soto et al., 2014) and melanoma (Fazakas et al., 2011). The cancer cell adhesion and transmigration processes are achieved through the interaction of metastatic cancer cells and the luminal surface of brain endothelial cells (Fazakas et al., 2011; Soto et al., 2014 and Jassam et al., 2015). CD15 and CD15s (sialyl CD15) are cell adhesion molecules, which have been shown to play a role in non-CNS cancer metastasis. CD15 is correlated with adhesion in lung cancer, by homophilic binding with CD15, which is expressed on endothelial cells and heterophilicly with other cell adhesion molecules such as the selectins (Martin et al., 1995, Brooks and Leatham, 1995, Jassam et al., 2015). CD15s is also a ligand for E-selectin and P-selectin (Polley et al., 1991) and is involved in adhesion process between cancer and endothelial cells (Giavazzi et al., 1993, Paschos et al., 2010). CD15s also mediates the initial tethering and rolling of white blood cells (WBCs) during homing process (Mondal et al., 2015). Despite the clinical importance of metastasis from lung to the brain, little is known concerning the interaction of NSCLC cell with brain endothelial cells. Although non-leukocyte-like mechanisms have been suggested in the
extravasation process of some cancer cells (Miles et al., 2008 and Martinez et al., 2013), the transmigration of cancer cells particularly to the brain has not been thoroughly investigated. It has been reported that melanoma cells are able to cross the endothelial cell monolayer as determined by measuring the transendothelial electric resistance (TEER) values which is an indicator of BBB functioning (Fazakas et al., 2011). Indeed long term co-culture of melanoma and brain endothelial cells shows a significant increase in permeability of the endothelial monolayer, as indicated by a discontinuous staining of both tight junction protein (ZO-1) as well as claudin and in the formation of ruptures in the endothelial monolayer (reviewed by Wilhelm et al., 2013).

This part of the project sheds light on the role of CD15 and CD15s in adhesion and transendothelial migration ability of NSCLC. Our findings suggest that overexpression of CD15 and CD15s increase the adhesion and transmigration of NSCLC cells during metastasis into the brain.
4.2 A strong correlation between genetic manipulation of \textit{FUT4} regulation and CD15 expression

Four different cell lines: SEBTA-001, NCI-H1299, COR-L105 and UP-007 were transfected with either a full-length \textit{FUT4-GFP} for CD15-overexpression or four \textit{FUT4} unique 29 mer shRNA constructs for CD15-knowckdown. CD15 expression in transfected cells with \textit{FUT4} cDNA was increased while, no CD15 expression was noticed on cells that treated with \textit{FUT4}-shRNA. This was measured using immunocytochemistry, Western blotting and flow cytometry analysis (Figure 32 A, B and C). Results showed prominent bands between 110-95 kDa suggesting that overexpression of CD15 following transfection with \textit{FUT4} cDNA in SEBTA-001, NCI-H1299, COR-L105 and UP-007 cells, nearly the same amount of CD15 expression was seen in the wild type and cells that transfected with the vehicle control. Faint and weak bands were noted post transfection with shDNA of \textit{FUT4}, which revealed that \textit{FUT4}-knockdown decreased the expression of CD15 downstream (Figure 32B). Flow cytometry results were consistent and showed similar trends in overexpression and knockdown, transfection of the \textit{FUT4} resulted in a significant increase (p<0.001) in CD15 expression in all cell lines (SEBTA-001, NCI-H1299, COR-L105 and UP-007) (Figure 32C). In SEBTA-001 CD15 increased from 54.7% to 92%, in NCI-H1299 increased from 76% to 90%, in COR-L105 increased from 23% to 89% and in UP-007 the increase was from 13.4% to 84% (Figure 32C, blue versus green). shRNA knockdow of \textit{FUT4} resulted in a significant decrease (p<0.001) in CD15 expression where SEBTA-001 showed only 9% positivity, in NCI-H1299 decreased to 8%, in COR-L105 decreased to 14% and to 6% in UP-007 cells (Figure 32C), (blue versus red). The findings revealed that genetic manipulation of \textit{FUT4} directly affected the expression of CD15 downstream. However, western blot results were not matching the flow cytometry results, and that may be caused by the protein precipitation in western blotting, which normally has no effect in flow cytometry analysis particularly, in cell membrane analysis.
Figure 32A: Localization of CD15 expression in FUT4-(cDNA and shRNA) transfected cells. Target cells (SEBTA-001, NCI-H1299, COR-L105 and UP-007) were transfected either with plasmids containing FUT4-cDNA to induce CD15 overexpression or four FUT4-shRNA constructs targeting FUT4 to inhibit the expression of CD15 downstream. Cells were grown on coverslips, fixed and extracellular immunostaining was performed to detect CD15 expression (red). All vectors were GFP. Scale bar=20μm.
Figure 32B: Representative Western blots show the levels of CD15 expression on cell membrane lysates. Transfected cells were lysed and cell membrane was extracted then Western blot was performed. No treatment was applied to wild type controls and vehicle controls were transfected with non-coding plasmids. All vectors were tagged with GFP gene.
Figure 32C: Representative flow cytometric analysis of CD15 expression in FUT4-(cDNA and shRNA) transfected cells. The histograms show percentages of CD15 positive cells in: wild type controls (blue) where no treatment applied, vehicle controls were transfected with non-coding plasmid (black), cells transfected with shRNA for FUT4 (red) and in cells transfection with cDNA for FUT4 (green). Data presented are mean±SE. Statistical significance is assessed by one-way ANOVA and Tukey’s post-hoc test, *(P<0.05), ** (P<0.01) difference compared to the wild type. Results are representative of three independent experiments carried out in triplicate. (N=3).
4.3 A strong correlation between genetic manipulation of FUT7 regulation and CD15s expression

CD15s expression was over expressed and knocked down by genetically manipulating the FUT7 in the following cell lines: SEBTA-001, NCI-H1299, COR-L105 and UP-007. ICC, Western blot and flow cytometry analysis were used to assess the expression of CD15s downstream. A prominent immunostaining of CD15s on the surface of the transfected cells with FUT7-cDNA. CD15s was evenly distributed across the cell body with intense staining on the edges of the cells, particularly at cell-cell junctions and adhesion sites. The absence of CD15s was noted in cells which were treated with FUT7-shRNA. All the transfected cells expressed GFP which confirmed the efficacy of transfection (Figure 33A). Western blotting results showed that bands between 180-100 kDa were identified suggesting that CD15s was highly expressed in cells which were transfected with FUT7-cDNA. Faint bands were noticed post-transfection with FUT7-shRNA indicating that knockdown had significantly decreased CD15s expression (Figure 33B). Results of flow cytometry analysis were consistent with ICC and WB results. CD15s expression was increased from 51% to 88% in SEBTA-001, from 47% to 78% in NCI-H1299, from 26% to 53.8% in COR-L105 and from 16.4% to 45% in UP-007 (Figure 33C) (blue line versus green line). Expression of CD15s was significantly decreased post-transfection with FUT4-shRNA in all the cell lines: in SEBTA-001 decreased to 12%, in NCI-H1299 decreased to 10%, COR-L105 show 13.5% positive and UP-007 were 9.3%. The data show that genetic manipulation of FUT7 affects the expression of CD15s in the treated cell lines (Figure 33C). There is a slight difference between western blot results and flow cytometry, which may be related to the effect of cellular precipitated proteins in western blotting, which has no effect in flow cytometry analysis.
Figure 33A: Localization of CD15s expression in FUT7-(cDNA and shRNA) transfected cells.

Target cells (SEBTA-001, NCI-H1299, COR-L105 and UP-007) were transfected either with plasmids containing FUT7-cDNA to induce CD15s overexpression or four FUT7-shRNA constructs targeting FUT7 to inhibit the expression of CD15s downstream. Cells were grown on coverslips, fixed and extracellular immunostaining was performed to detect CD15s expression (red). All vectors were GFP-tagged. Scale bar=20µm.
Figure 33B: Representative Western blots show expression of CD15s post-transfection with (cDNA and shRNA)-FUT7. CD15s levels were confirmed using Western blotting and showed the effect of genetic manipulation of FUT7 on the expression of CD15s. ABCE1 protein was used as a loading control. No treatment was applied to wild type controls. Vehicle controls were transfected with non-coding plasmids.
Figure 33C: Representative flow cytometric analysis of CD15s expression in FUT7-(cDNA and shRNA) transfected cells. The histograms show percentages of CD15s positive cells in: wild type controls (blue) where no treatment applied, vehicle controls were transfected with non-coding plasmid (black), cells transfected with shRNA for FUT7 (red) and in cells transfection with cDNA for FUT7 (green). Data presented are mean±SE. Statistical significance assessed by one-way ANOVA and Tukey’s post-hoc test, *(P<0.05), ** (P<0.01) difference compared to the wild type. Results are representative of three independent experiments carried out in triplicate. (N=3).
4.4 CD15 expression correlates with cancer cell adhesion

Following genetic manipulation of FUT4/CD15, cancer cell adhesion to a monolayer of human brain endothelial cells (hCMEC/D3) was investigated. In general, metastatic lung to brain cancer cells (SEBTA-001) and metastatic lung to lymph node cells (NCI-H1299) showed the highest number of adherent cells whereas lower adhesion was seen in primary lung cancer cells (COR-L105) and GBM cells (UP-007) (Figure 34A). There was no statistical difference in adhesion potential which was observed in cells transfected with vehicle control in all cell lines. Following the overexpression of FUT4/CD15 in all the cell lines (SEBTA-001, NCI-H1299, COR-L105 and UP-007), a significant increase in adhesion (2 fold) was seen compared to the adhesion of wild type controls of the same cell line. Adhesion of cancer cells was significantly reduced following the knockdown of FUT7/CD15 and the number of adherent cells were less compared to the wild type (P<0.001) in all cell lines studied (SEBTA-001, NCI-H1299, COR-L105 and UP-007) (Figure 34 A) (blue versus green).
Figure 34A: Quantitative adhesion of cancer cells following the genetic manipulation of FUT4/CD15.

Figure 34 A. Quantitative adhesion of cancer cells following the genetic manipulation of FUT4/CD15. Adhesion of Primary (COR-L105), metastatic NSCLCs (SEBTA-001 and NCI-H1299) and GBM (UP-007) cells were assessed. Cancer cells were incubated for 90 minutes on a monolayer of activated brain endothelial cells (hCMEC/D3). Non-adherent cancer cells were washed off then cells were lysed and quantified via a microplate reader at 480–520 nm. Graphs show that cancer cell adhesion was significantly decreased following FUT4/CD15 knockdown (red bars). Overexpression of FUT4/CD15 increased adhesion of cancer cells (green bars) compared with wild type control, (**P<0.001). N= 3.
4.5 CD15s expression correlates with cancer cell adhesion

After genetic manipulation of FUT7/CD15s, adhesion of cancer cells to brain endothelial cells (hCMEC/D3) was assessed. Metastatic lung to brain cancer cells (SEBTA-001) and metastatic lung to lymph node cells (NCI-H1299) showed the highest number of adherent cells whereas lower adhesion was seen in primary lung cancer cells (COR-L105) and GBM cells (UP-007) (Figure 34 B). There was no statistical difference in adhesion potential which was observed in cells transfected with vehicle control in all cell lines. Following the overexpression of FUT7/CD15s in all the cell lines (SEBTA-001, NCI-H1299, COR-L105 and UP-007), a significant increase in adhesion (2 fold) was seen compared to the adhesion of wild type controls of the same cell line. A significant reduction was observed in the number of adherent cancer cells following the knockdown of FUT7/CD15s in SEBTA-001 by almost 2.5 fold decrease in adhesion ability followed by a 2.7 fold decrease in NCI-H1299, a 2.8 fold decrease in COR-L105 and a 2.8 fold decrease in UP-007 cells compared to the wild type (P<0.001) (Figure 34 B) (blue versus green).
Figure 34 B: Quantitative adhesion of cancer cells following the genetic manipulation of FUT7/CD15s. Adhesion of Primary (COR-L105), metastatic NSCLCs (SEBTA-001 and NCI-H1299) and GBM (UP-007) cells were assessed. Cancer cells were incubated for 90 minutes on a monolayer of activated brain endothelial cells (hCMEC/D3). Non-adherent cancer cells were washed off then cells were lysed and quantified via a microplate reader at 480–520 nm. Graphs show that cancer cell adhesion was significantly decreased following FUT7/CD15s knockdown (red bars). Overexpression of FUT7/CD15s increased adhesion of cancer cells (green bars) compared with wild type control, (***P<0.001). N= 3.
4.6 Different transendothelial migration ability among different cancer cells

To investigate the transendothelial migration ability of target cells (SEBTA-001, NCI-H1299, COR-L105 and UP-007), two assays were used: EVOM and ECIS. In both assays human brain endothelial cells (hCMEC/D3 were grown on a fibronectin (10µg/mL) coated surface. Cancer cells were added on top of the monolayer after the TEER value readings plateaued. The integrity of endothelial membrane was then monitored. EVOM results showed a significant (p<0.01) and accelerated decrease in TEER value from 28.9 to 15.1 Ω.cm² within 24 hours following SEBTA-001 cells addition. This indicates that cancer cells disrupted the endothelial membrane in attempt to cross the brain endothelial monolayer. A decrease in TEER value was also seen after NCI-H1299 cells were added from 28.9 to 20.0 Ω.cm² however, NCI-H1299 took longer to decrease the resistance of the endothelial barrier compared to SEBTA-001 cells. No significant reduction in the transendothelial electrical resistance of brain endothelial monolayer was observed after addition of primary lung cancer cells (COR-L105) with resistance (TEER) change from 28.9 Ω.cm² to 26.9 Ω.cm². Similarly, no decrease in TEER (28.9 Ω.cm²) induced by addition of GBM cells (UP-007) on the endothelial monolayer. (Figure 35 A and B). Readings were compared to the control well (no cancer cells added/ only monolayer of brain endothelial cells). The histograms generated by ECIS showed the resistance of the endothelial monolayer per time point. Results showed a decrease in TEER value (51%) post addition of SEBTA-001 cells compared to the control. Post addition of NCI-H1299 cells caused a 42% decrease in TEER compared to control. These findings indicated that the endothelial barrier was disrupted by the secondary (metastatic) lung cancer cells. While, addition of primary lung cancer cells (COR-L105) did not cause a decrease in the resistance of the hCMEC/D3 barrier (only 4% decrease compared to the control). These results suggest that no disruption was induced by COR-L105 cells. Interestingly, an increase in resistance was observed following the addition of UP-007 cells suggesting that GBM cells were not able to cross the brain endothelial monolayer (Figure 35C).
Figure 35 (A-B): Changes in transendothelial electrical resistance (TEER) in the presence of cancer cells measured by EVOM. (hCMEC/D3) brain endothelial cells were grown on semipermeable filters (8 μm pore size). TEER values were pre and post-addition of cancer cells using EVOM. A: Graph showed a decreased TEER induced by SEBTA-001 and NCI-H1299 cells, only a small decrease in resistance was seen after addition of primary lung cancer cells (COR-L105). Addition of UP-007 however, increased the resistance of hCMEC/D3 (EC). B: Quantification of TEER at 24 hours post-addition of cancer cells. N=3.
Figure 35C: Representative ECIS graphs showing changes in TEER following addition of cancer cells. Endothelial cells were seeded on gold electrode and TEER values were recorded in a real time manner using electric cell-substrate impedance sensing (ECIS). Results showed an increase in TEER before addition of cancer cells followed by a significant and accelerated decrease in TEER after addition of SEBTA-001 and NCI-H1299 cells. These findings confirmed potential disruption in endothelial monolayer caused by the added cancer cells. No significant difference was observed after addition of primary lung cancer cell (COR-L105) and GBM (UP-007) cells. N=3.
4.7 Cancer cell transendothelial migration was increased by overexpression of
FUT4/CD15 and inhibited by FUT4/CD15 knockdown

To investigate whether overexpression or knockdown of FUT4/CD15 may play a role in
transendothelial migration of cancer cells during the early stages of brain metastasis, the
changes in transendothelial electric resistance (TEER) of the brain endothelial monolayer
was followed using three approaches: EVOM, ECIS™ and CellZscope®. The findings of
EVOM showed a remarkable decrease in TEER value within few hours following the
addition of wild type SEBTA-001 (18.0 Ω.cm² compared to control 24.8 Ω.cm²) and NCI-
H1299 (19.2 Ω.cm²) compared to control where no cancer cells were added (24.8 Ω.cm²).
A higher and more accelerated decrease in TEER was seen following addition of cancer
cells overexpressing FUT4/CD15 from 24.0 Ω.cm² to 15.6 Ω.cm² in SEBTA-001 and 24.0
Ω.cm² to 16.1 Ω.cm² in NCI-H1299 compared to control. No significant changes were
noted in presence of FUT4/CD15 knocked-down cells: SEBTA-001 (24.8 Ω.cm²) and
NCI-H1299 (24.4 Ω.cm²). These findings indicated that knockdowned cells were not been
able to disrupt the brain endothelial monolayer (Figure 4A). No changes were observed
after addition of COR-L105 TEER: (23.0 Ω.cm²) and UP-007 (22.9 Ω.cm²) in wild type
and FUT4/CD15 knockdown clones. A decline in TEER value was caused by
overexpression of FUT4/CD15, where COR-L105 overexpressing CD15 decreased the
resistance from 23.0 Ω.cm² to 18.0 Ω.cm² and UP-007 to from 23.0 Ω.cm² to 18.4 Ω.cm²
after 24 hours (Figure 36 A).

ECIS results were consistent with EVOM findings. A strong correlation was noted
between the expression of FUT4/CD15 and the decrease in resistance (TEER) of brain
endothelial monolayer. Overexpression of FUT4/CD15 was seen to be correlated with
decrease in resistance (TEER) in all cell lines compared to the wild type controls and
existence of overexpressing cells decreased TEER: SEBTA-001 (63%), NCI-H1299
(61%), COR-L105 (60.8%) and UP-007 (58%) and knockdown of FUT4/CD15 showed
only a slight decrease in TEER values: SEBTA-001 (3%), NCI-H1299 (1.5%), COR-L105 (0.5%) and UP-007 (6%) (Figure 36 B).

CellZScope® studies showed that overexpression of FUT4/CD15 was correlated with a decrease in TEER value after 3 to 5 hours post-addition of cancer cells: SEBTA-001 induced a 65% decrease, NCI-H1299 68% decrease, COR-L105 60% decrease and UP-007 66% decrease compared to the control where no cancer cells were added. Knockdown of FUT4/CD15 only showed a slight decrease in resistance value: SEBTA-001 (2%) NCI-H1299 (2.8%), COR-L105 (1.6%) and UP-007 (0.5%) compared to control (Figure 36 C).
Figure 36 A page 1. Effect of overexpression and knockdown of FUT4/CD15 on SEBTA-001 and NCI-H1299 cells transendothelial migration by EVOM.

Figure 36 A page 1. Effect of overexpression and knockdown of FUT4/CD15 on SEBTA-001 and NCI-H1299 cells transendothelial migration. Graphs show that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. hCMEC/D3 were grown on transwell filters with 8µm pore size. Resistance values were manually measured using EVOM. Cancer cells were added at 1x10^4/well. The green line shows the control well where no cancer cells were added, red line refers to cell lines overexpressing CD15, blue line represents adding cell lines with CD15s knockdown, yellow line refers to the addition of the wild type and the black line represents the blank control. (EC): hCMEC/D3 cells, N=3.
Figure 36 A page 2. Effect of overexpression and knockdown of FUT4/CD15 on cancer cell (COR-L105 and UP-007) transendothelial migration by EVOM.

Graphs show that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. hCMEC/D3 were grown on transwell filters with 8µm pore size. Resistance values were manually measured using EVOM. Cancer cells were added at 1x10^4/well. The green line shows the control well where no cancer cells were added, red line refers to cell lines overexpressing CD15, blue line represents adding cell lines with CD15 knockdown, yellow line refers to the addition of the wild type and the black line represents the blank control. (EC): hCMEC/D3 cells, N=3.
Figure 36 B page 1. Effect of overexpression and knockdown of FUT4/CD15 on SEBTA-001 cells transendothelial migration by ECIS. Results show that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10^4/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15, blue: cancer cells with CD15 knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. (*P<0.01), N=3.
Figure 36 B page 2. Effect of overexpression and knockdown of FUT4/CD15 on NCI-H1299 cells transendothelial migration by ECIS.

The graph shows that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (EC)(hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10^4/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15, blue: cancer cells with CD15 knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. (*P<0.01), N=3.
Figure 36 B page 3. Effect of overexpression and knockdown of FUT4/CD15 on COR-L105 cells transendothelial migration by ECIS. The graph show that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (EC) (hCMEC/D3) were grown on gold electrodes. Cancer cells were added at $1 \times 10^4$/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15, blue: cancer cells with CD15 knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. (*P<0.01), N=3.
Figure 36 B page 4. Effect of overexpression and knockdown of FUT4/CD15 on UP-007 cells transendothelial migration by ECIS.

The graph shows that transendothelial migration was increased by overexpression of FUT4/CD15 but inhibited by FUT4/CD15 knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (EC)(hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10⁴/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15, blue: cancer cells with CD15 knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. (*P<0.01), N=3.
Figure 36C page 1: Effect of FUT4/CD15 genetic manipulation on SEBAT-001 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of SEBTA-001 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15, blue: cells with CD15 knockdown, yellow: the wild type and black represents the blank control. (EC): hCMEC/D3 cells, (*P<0.01), N=3.
Figure 36 C page 2: Effect of FUT4/CD15 genetic manipulation on NCI-H1299 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of SEBTA-001 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15, blue: cells with CD15 knockdown, yellow: the wild type and black represents the blank control. (EC): hCMEC/D3 cells, (*P<0.01), N=3.
Figure 36C page 3: Effect of FUT4/CD15 genetic manipulation on COR-L105 cells transendothelial migration by CellZscope.

Figure 36C page 3: Effect of FUT4/CD15 genetic manipulation on COR-L105 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of SEBTA-001 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15, blue: cells with CD15 knockdown, yellow: the wild type and black represents the blank control(EC): hCMEC/D3 cells, (*P<0.01), N=3.
Figure 36 C page 4: Effect of FUT4/CD15 genetic manipulation on UP-007 cells transendothelial migration by CellZscope.

Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of SEBTA-001 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15, blue: cells with CD15 knockdown, yellow: the wild type and black represents the blank control. hCMEC/D3 cells (EC), (*P<0.01), N=3.
4.8 Increase in transendothelial migration of cancer cells was induced by overexpression of FUT7/CD15s and blocked by FUT7/CD15s knockdown.

To investigate the effect of overexpression or knockdown of FUT7/CD15s may play a role in transendothelial migration of cancer cells during seeding into the brain, the changes in transendothelial electric resistance (TEER) of the brain endothelial monolayer was assessed using three approaches: EVOM, ECIS™ and CellZscope®. Knockdown and overexpression of FUT7/CD15s were induced in the following cell lines: SEBTA-001, NCI-H1299, COR-L105 and UP-007. Cancer cells were added on top of the hCMEC/D3 monolayer and then the changes in TEER values of brain endothelial cells monolayer was measured. EVOM results showed a significant and accelerated decrease in TEER value of brain endothelial monolayer post addition of the wild type of metastatic lung cancer cells (SEBTA-001) as it decreased the TEER to 16.9 Ω.cm² comparing to control (24.5 Ω.cm²) and NCI-H1299 reduced the resistance to 19.0 Ω.cm² compared to control 24.5 Ω.cm². A similar decline was observed when FUT7/CD15s-overexpressing cells were added where SEBTA-001 decreased the TEER to 14.9 Ω.cm² and NCI-H1299 to 19.0 Ω.cm² compared to the control (24.5 Ω.cm²). Whereas, no decrease was detected after addition of FUT7/CD15s knockdowns: SEBTA-001 (25.0 Ω.cm²) and NCI-H1299 (25.1 Ω.cm²). No decrease was observed in TEER value even after 24 hours post addition of the wild type of both, COR-L105 (22.0 Ω.cm²) and UP-007 (25.3 Ω.cm²), the same behaviour was observed in their clones that were treated with shRNA for FUT7/CD15s. No significant decrease in TEER values COR-L105: (22.0 Ω.cm²) and (UP-007: 24.9 Ω.cm²). Interestingly, overexpression of FUT7/CD15s in COR-L105 and UP-007 significantly decreased TEER values as COR-L105: 17.0 Ω.cm² compared to the control: 24.5 Ω.cm² and UP-007: 18.4 Ω.cm² compared to control: 24.5 Ω.cm² (Figure 37A). ECIS analysis findings (Figure 37B) showed that a prominent decrease in the electric resistance of the brain endothelial barrier was correlated with FUT7/CD15s overexpression in all the cell lines: a 69% decrease was caused by addition of SEBTA-001 and a 56% decrease in
resistance caused by the wild type compared to control where no cancer cells were added, a 61% decrease caused by NCI-H1299 and 43% decrease induced by its wild type compared to control, a 60.8% decrease was induced by COR-L107 compared to non-significant decreased caused by its wild type and finally, UP-007 overexpressing FUT7/CD15s cells were seen to decrease the resistance with 54% compared to the control and knockdown of FUT7/CD15s was correlated with high and constant resistance of the hCMEC/D3 monolayer which suggested a decrease in transendothelial migration of cancer cells. CellZscope® results were consistent with the results of EVOM and ECIS, a noticeable and fast decrease in TEER value was recorded post addition of SEBTA-001 within 5 hours and 7 hours (53%) decreased in resistance compared to control where no cancer cells were added, NCI-H1299 caused a 54.6% decrease comparing to control. An even faster and sharper decrease was caused by SEBTA-001(58% decrease compared to control) and NCI-H1299 (55.4% decrease compared to control) cells treated for FUT7/CD15s overexpression. However, the brain endothelial barrier was seen to maintain its integrity and high TEER value even after adding the highly metastatic lung cancer cells SEBTA-001 (3% decrease) and NCI-H1299 cells (1% decrease) that were treated to knockdown FUT7/CD15s. No significant decrease was recorded after adding COR-L105 and UP-007 (6% increase comparing to control) cells similar to the behaviour of its FUT7/CD15s knockdown clones, whereas a significant decrease was caused by overexpression of FUT7/CD15s in the same cells as a 60% decrease in TEET followed addition of COR-L105 and a 66% decrease in TEER caused by UP-007 (Figure 37C).
Figure 37 A page 1. Effect of overexpression and knockdown of FUT7/CD15s on cancer cell (SEBTA-001 and NCI-H1299) transendothelial migration measured by EVOM.

Figure 37 A page 1. Effect of overexpression and knockdown of FUT7/CD15s on cancer cell (SEBTA-001 and NCI-H1299) transendothelial migration. Graphs show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. hCMEC/D3 (EC) were grown on transwell filters with 8µm pore size. Resistance values were manually measured using EVOM. Cancer cells were added at 1x10^4/well. The green line shows the control well where no cancer cells were added, red line refers to cell lines overexpressing CD15s, blue line represents adding cell lines with CD15s knockdown, yellow line refers to the addition of the wild type and the black line represents the blank control. N=3.
Figure 37A page 2. Effect of overexpression and knockdown of FUT7/CD15s on cancer cell (COR-L105 and UP-007) transendothelial migration. Graphs show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. hCMEC/D3 (EC) were grown on transwell filters with 8µm pore size. Resistance values were manually measured using EVOM. Cancer cells were added at 1x10^4/well. The green line shows the control well where no cancer cells were added, red line refers to cell lines overexpressing CD15s, blue line represents adding cell lines with CD15s knockdown, yellow line refers to the addition of the wild type and the black line represents the blank control. N=3.
Figure 37 B page 1. Effect of overexpression and knockdown of FUT7/CD15s on SEBTA-001 cells transendothelial migration by ECIS. Results show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (hCMEC/D3) (EC) were grown on gold electrodes. Cancer cells were added at 1x10^4/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15s, blue: cancer cells with CD15s knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 page 2. Effect of overexpression and knockdown of FUT7/CD15s on NCI-H1299 cells transendothelial migration by ECIS. Results show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (EC) (hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10^4/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15s, blue: cancer cells with CD15s knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 B page 3. Effect of overexpression and knockdown of FUT7/CD15s on COR-L105 cells transendothelial migration by ECIS. Results show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. Resistance of hCMEC/D3 barrier was measured and recorded in real time. Brain endothelial cells (EC)(hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10^4 /well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15s, blue: cancer cells with CD15s knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 B page 4. Effect of overexpression and knockdown of FUT7/CD15s on UP-007 cells transendothelial migration by ECIS. Results show that transendothelial migration was increased by overexpression of FUT7/CD15s but inhibited by FUT7/CD15s knockdown. Resistance of hCMEC/D3 (EC) barrier was measured and recorded in real time. Brain endothelial cells (hCMEC/D3) were grown on gold electrodes. Cancer cells were added at 1x10^4/well. Green line indicates where no cancer cells were added, red: cancer cells overexpressed CD15s, blue: cancer cells with CD15s knocked down, yellow: wild type cells and black line: blank. The lower panel shows representative images of the endothelial monolayer after 24 hours post-addition of cancer cells. The image was obtained using a X4 objectives via a phase contrast microscope. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 C page 1: Effect of FUT7/CD15s genetic manipulation on SEBTA-001 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of SEBTA-001 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15s, blue: cells with CD15s knockdown, yellow: the wild type and black represents the blank control. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 C page 2: Effect of FUT7/CD15s genetic manipulation on NCI-H1299 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of NCI-H1299 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15s, blue: cells with CD15s knockdown, yellow: the wild type and black represents the blank control. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 C page 3: Effect of FUT7/CD15s genetic manipulation on COR-L105 cells transendothelial migration by CellZscope. Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of COR-L105 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15s, blue: cells with CD15s knockdown, yellow: the wild type and black represents the blank control. hCMEC/D3 cells (EC), (*P<0.01), N=3.
Figure 37 C page 4: Effect of FUT7/CD15s genetic manipulation on UP-007 cells transendothelial migration by CellZscope. Cancer cells added.

Histogram shows the changes in TEER value using CellZscope automated system, pre and post addition of UP-007 cells, the green represents control well where no cancer cells were added, red line: cell were overexpressing CD15s, blue: cells with CD15s knockdown, yellow: the wild type and black represents the blank control. hCMEC/D3 cells (EC), (*P<0.01), N=3.
4.9 Discussion

The brain is known to be a key target for metastasis cancers from the lung. 20%-40% of patients with NSCLC cancer have been reported to develop secondary brain tumours (Ali et al., 2013). Brain metastasis is correlates with poor prognosis. The mechanisms underlining brain metastasis are not fully elucidated. The metastatic process includes a complex interaction between metastatic cancer cell and host environment. There are five key elements in the process of metastasis to the brain: (a) intravasation from within the primary cancer (b) travelling within the blood circulation (c) adhesion to the target organ (brain) vascular endothelium (d) extravasation into brain tissue and (e) colonisation and growth within the brain. Cancer cells generally metastasise to the brain via the capillaries which constitute a compartment of the BBB vascular network. The importance of brain endothelial cells in brain metastasis has recently been highlighted; particularly in the early stages of cancer cell seeding (Soto et al., 2014). CD15 and CD15s are important adhesion molecules and both involved in the homing process of leukocytes (Strell and Entschladen, 2008). Their overexpression has been correlated with cancer progression and metastasis in non-CNS cancers CD15 (Gout et al., 2006) and CD15s (Desiderio et al., 2015) were seen to accumulate at the invading edges of tumours. Little is known about these molecules within the CNS and even less is known about their role in brain metastasis. In a previous study, we showed that CD15 plays a key role in adhesion of cancer cells to brain endothelium via CD15-CD62E interaction and adhesion of cancer cells was significantly reduced by immune-blocking of CD15 (Jassam et al., 2015). Here, we investigated the role of knockdown and overexpression of CD15 and CD15s in adhesion on brain endothelium as well as their role in transmigration across a brain endothelial monolayer. A human microvascular endothelial cell line (hCMEC/D3) was used to represent the first line in within an in vitro BBB model to circumvent the use of experimental animals and gain important information within the metastatic process. In vivo models have been shown to be flawed for the study of human cerebral metastasis due to the species differences in BBB
(Syvanen et al., 2009). Human brain endothelial monolayers in vitro models have previously been used to study mechanism involved in transendothelial migration of melanoma cells (Fazakas et al., 2011) and transmigration of lymphocytes (Kugler et al., 2007 and Man et al., 2008). In vitro BBB models are used to study, one side of metastasis such as cell-cell interactions. However, there are many limitations and challenges face those models. Such as lack of desirable shear stress, use of non-transformed cells, and the accurate and functional co-cultures representing the accurate BBB designed. The hCMEC/D3 cell line has been widely used in in vitro human BBB model lines (Forster et al., 2008 and Carl et al., 2010). CD15 and CD15s biosynthesis is widely known to be regulated by fucosyltransferase enzymes (α1,3 FUTs); FUT3, -4, -5, -6, -7 and -9. One or more of FUTs enzymes are involved in CD15 and CD15s biosynthesis depending on tissue type (de Vries et al., 2001). It was further shown that FUT4 and FUT7 are predominantly expresses in the haematopoietic cells and overexpression of fucosyltransferase IV (FUT4) increases the expression of CD15 which correlates with increased metastasis in colorectal cancer (Giordano et al., 2015)33. Conversely, FUT4 knockdown reduced the expression of CD15 in promyelocytes and monocytes but not in mature granulocytes (Nakayama et al., 2001). It was also reported that FUT4 and FUT7 knockdown was shown to significantly reduce the homing of leukocytes and lymphocytes (Maly et al., 1996 and Homeister et al., 2001). Our results showed that the highest expression level of FUT4/CD15 and FUT7/CD15s was seen in metastatic lung cancer cells (SEBTA-001 and NCI-H1299) compared to primary lung cancer and GBM cells. These findings are in concert with previous studies where it was shown that fucosyltransferase (FUT) overexpression in general was correlated with poor prognosis and metastasis in prostate cancer (Jorgensen et al., 1995 and Barthel et al., 2004) as well as lung cancer (Ogawa et al., 1996). It was reported that colon carcinoma cells overexpressing CD15s were able to metastasise to the liver compared to cells with low levels of CD15s (Izumi et al., 1995) in in vivo models. FUTs have also been reported to regulate the homing of metastatic prostate cancer cells in
mice (Barthel et al., 2009). Overexpression of the fucosyltransferase VII (FUT7) enzyme via upregulation of the FUT7 gene was seen to increase the expression of CD15s in prostate cancer cells (Barthel et al., 2009) and FUT7 knockdown downregulated the expression of CD15s and led to CD15s deficiency in human promyelocytic leukaemia cells (Weston et al., 1999). In our study, FUT4 and FUT7 were chosen to manipulate the expression of CD15 and CD15s by overexpressing and silencing respectively. Transfection with FUT4 and FUT7 genes were applied in four different cancer cell lines (SEBTA-001, NCI-H1299, COR-L105 and UP-007). Results showed that the FUT4 gene directly controlled the expression of CD15 and the same scenario was seen during transfection with the FUT7 gene which induced the overexpression of CD15s. These findings agreed with what those previously reported where CD15 (Lewis x) was mainly controlled by FUT4 and FUT9 at the organ buds progressing in mesenchymal of human embryos (Cailleau-Thomas et al., 2000). We also found that silencing FUT4 and FUT7 genes led to a downregulation of the expression of CD15 and CD15s respectively in all the studied cancer cell lines. The same effect was previously reported with FUT7 knockdown on reduced expression of CD15s in lymphocyte cells (Buffone et al., 2013). Another study showed that silencing of ST3Gal-4 reduced the biosynthesis of CD15s in cells of human myeloid lineage (Mondal et al., 2015). There is little known about FUTs enzymes and their functional role in lung cancer or GBM cells thus, we decided to start with FUT4 and FUT7. ICC, WB and flow cytometry analysis were employed to confirm expression levels of CD15 and CD15s post FUT4/CD15 and FUT7/CD15s knockdown and overexpression. The effect of knockdown and overexpression of FUT4/CD15 and FUT7/CD15s were investigated on the adhesion ability of cancer cells. Overexpression of FUT4/CD15 was seen to increase the adhesion ability in all studied cancer cells and FUT4/CD15 knockdown was seen to decrease the adhesion of cancer cells. These findings are in agreement with those of Yang et al., 2013 who highlighted that overexpression of FUT4 correlated with high metastatic potential in breast cancer cells and is implicated in both adhesion and invasion of breast cancer cells.
Fucosyltransferase (FUT4) protein was seen to be overexpressed on highly invasive cells (Yang et al., 2012). Overexpression of FUT4 and FUT7 were also found to be correlated with metastasis of lung cancer cells. Similarly, we observed that overexpression of \textit{FUT7}/CD15s increased adhesion of cancer cells and knockdown of \textit{FUT7}/CD15s significantly reduced the adhesion ability of cancer cell to brain endothelial cells. It was reported that silencing of \textit{FUT7}/CD15s reduced the adhesion ability of human leukocytes (Buffone et al., 2013).

Adhesion of cancer cells is an important step in cancer metastasis but do not mean that all adherent cells will be able to cross the BBB into the brain therefore we investigated transendothelial migration ability of cancer cells, by measuring the TEER value of hCMEC/D3 barrier using three different approaches: the first one was EVOM, which is based on applying a non-invasive current (AC), it is useful in providing accurate readings and simple to use however, it is not helpful in providing real-time TEER readings that is why we decided to use the other two automated devices: ECIS and CellZscope. Both two approaches are based on applying a non-invasive current (AC) to detect the gaps between the attached cells; however, ECIS highly sensitive device which, is designed in a way that the mobility of the adherent cells may affect the resistance readings and to avoid this we validate ECIS results by using another device. CellZscope is perfectly designed to measure the resistance and mathematically calculates the data and then displays it as a pure TEER values. Our results showed that trans-endothelial migration ability of cancer cells was increased following overexpression of \textit{FUT4}/CD15 and significantly decreased in knockdown cells. These results suggested that FUT4/CD15 play a role in transendothelial migration of cancer cells during metastasis to the brain. Likewise, \textit{FUT7}/CD15s silencing clearly reduced the ability of cancer cells to transmigrate via the brain endothelial monolayer as seen by no decrease in the resistance (TEER) value. These findings highlight that \textit{FUT7}/CD15s may also play a role in NSCLC transendothelial migration. These
findings were supported by previous literature which highlighted that knockdown of
FUT7/CD15s reduced the transendothelial migration of human leukocytes (Buffone et al., 2013). An in vivo study also showed that decreasing CD15s biosynthesis via a disaccharide
compound significantly reduced the metastasis of lung carcinoma cells (Brown et al.,
2009). This study demonstrated the key role of CD15 and CD15s in adhesion and
transendothelial migration.

In conclusion, our findings showed a strong correlation between FUT4/CD15 and
FUT7/CD15s in adhesion of lung cancer cells and transendothelial migration potential.
Knockdown of FUT4/CD15 and FUT7/CD15s significantly reduced the number of
adherent metastatic and primary lung cancer cells as well as GBM cells to brain
endothelium. Similarly reduced transendothelial migration ability of primary lung cancer
and GBM cells were observed following silencing of CD15 and CD15s. Highly metastatic
lung cancer cells (SEBTA-001 and NCI-H1299) showed similar results. TEER values of
brain endothelial monolayer did not decrease following knockdown of (–FUT4 or –FUT7)
cancer cells. In parallel transfection of (+FUT4 or +FUT7) in cancer cells showed high
adhesive potential and transendothelial migration ability via the brain endothelial
monolayer.
CHAPTER FIVE

The expression of CD15 and CD15s during different stages of cell cycle in cancer cells
5.1 Expression of CD15 and CD15s is correlated with cell cycle arrest at G1 phase in primary and metastatic brain tumour cells

The expression of cell membrane glycoconjugates is reported to be highly influenced by cell differentiation status (Hakomori 1981 and Stamatos et al., 2010). Some cancer cells express particular glycoconjugates during the transformation process and the overexpression of some of these conjugates has been directly correlated with malignancy (Schultz et al., 2013). CD15s has been shown to play a key role in cell-cell recognition as in sperm-egg fertilisation (Tulsiani and Abou-Haila, 2012) and in extravasation of leukocytes (Buffone et al., 2013). Both CD15 and CD15s are expressed in a variety of non-neoplastic cells such as granulocytes, monocytes (Nakayama et al., 2001), epithelial cells (Atsuta et al., 1997) and neurons (Mai and Reifenberger 1988; Chaoyang et al., 2007). A considerable number of studies have shown a strong correlation between overexpression of CD15, CD15s and malignancy in different cancers such as; Hodgkin’s lymphoma, breast, lung (Ohana-Malka et al., 2003, Brooks et al., 1995, Gadhoum et al., 2008 and renal carcinoma (Tozawa et al., 1995), colon (Rho et al., 2005), head and neck squamous carcinoma (Fukusumi et al., 2014) and brain metastases from lung (Notle et al., 2013) and breast (Gadhoum et al., 2008). Although CD15 was previously reported to be rarely expressed in anaplastic glioma and glioblastoma (Mai and Reifenberger; Martin et al., 1995) it has recently been suggested that CD15 may serve as a marker for brain cancer stem-like cells in human astrocytoma, ependymoma and medulloblastoma (Mao et al., 2009). The aim of this study was to gain greater understanding of CD15 and CD15s expression in CNS malignancies. Herein we demonstrated that expression of CD15 and CD15s is cell cycle dependent, which may account for the lack of consistency in previous studies.
5.2 Expression of CD15 in non-synchronised brain cancer cells

Under non-synchronised culture conditions, immunocytochemistry and confocal results indicate an absence of CD15 expression in non-neoplastic astrocytes (SC-1800), while in glioblastoma cells (SNB-19 and UP-007), expression of CD15 was noted on the cell surface of individual cells (Figure 38 A). Semi-quantitative analysis revealed a 5 fold higher number in CD15 fluorescence intensity in UP-007 cells and a 4 fold higher in SNB-19 cells compared to SC-1800 and isotype control (Figure 38 A, B; p< 0.05). CD15 expression in secondary lung to brain metastatic tumour cell lines demonstrated a 15 fold higher in SEBTA-001 and 12 fold higher in SEBTA-005 compared to SC-1800 and isotype control (Figure 38 A, B; p<0.01). Western blot analysis of CD15 expression in SC-1800 cells confirmed the confocal data showing no detectable level of CD15 (Figure 38 C). A CD15 immunoreactive protein at molecular weight 95-118 kDa was detected in both GBM cell lines (UP-007 and SNB-19) and lung to brain metastatic cells (SEBTA-001 and SEBTA-005). ABCE-1 with a molecular weight 68 kDa was used to ensure equal loading of samples (Figure 38 C). The level of CD15 expression in SEBTA 001 and SEBTA-005 is higher compared to the glioma cell lines which is in agreement with the ICC results. Flow cytometry analysis indicated that only 3% of SC-1800 expressed CD15 and this was not significantly different from isotype controls (Figure 38 E). CD15 was expressed at a lower level in SNB-19 (9.5%) and UP-007 (13.4%) compared to isotype control (p<0.05 and p<0.01) and to SEBTA-001(44%) and SEBTA-005 (36%) cells respectively (Figure 38 D and E). Both the lung to brain metastatic cell lines were significantly higher in terms of their CD15 positive cells compared to isotype controls (p<0.001) and the glioma cell lines (p<0.01).
Figure 38. Expression and localization of CD15 in non-synchronised cells.

A: Immunocytochemistry images showing no CD15 expression (green) in non-neoplastic astrocytes (SC-1800) but low positivity in primary GBM cells (UP-007 and SNB-19) and secondary brain cancer cells (SEBTA-001 and SEBTA-005). Hoechst blue was used as a nuclear counterstain. Scale bar =20μm. B: Semi-quantitative analysis of CD15 from confocal microscopy images using Zeiss Zen image software. C: Western blot analysis shows no CD15 expression in human astrocytes, weak expression in GBMs (UP-007 and SNB-19) and highest expression in brain secondary metastatic cancer cells (SEBTA-001 and SEBTA-005). D: Representative flow cytometric histograms and E: Quantitative flow cytometric analysis of CD15 expression in SC-1800, UP-007, SNB-19, SEBTA-001 and SEBTA-005. CD15 was highly expressed in SEBTA-001 and SEBTA-005 and least expressed in SC-1800. *(P≤0.05), ** (P<0.01) and *** (P<0.001) difference compared to the IgM isotype control. Results are representative of three independent experiments carried out in triplicate (n=3).
5.3 Expression of CD15s in non-synchronised brain cancer cells

CD15s was not detected on the surface of SC-1800 whereas a faint expression was detected in primary glioma cell lines with the highest expression seen in the lung to brain metastatic cell lines (Figure 39A). As with CD15, CD15s was localised to the outer edges of cell surface. Semi-quantitative analysis there was a 2.9 fold increase in CD15s expression in UP-007 and a 5.1 fold increase in SNB-19 cells compared to the isotype controls (Figure 39 A, B p<0.05). SEBTA-001 is 7 fold and SEBTA-005 is 6 fold higher in CD15s intensity compared to the expression of CD15s in SC-1800 and isotype control (Figure 39 A, B p<0.001). Western blot analysis (Figure 39 C) failed to detect CD15s protein in SC-1800 whereas a CD15s-immunoreactive proteins at molecular weight 95-118 kDa was detected in both GBM cell lines (UP-007 and SNB-19) and lung to brain metastatic cells (SEBTA-001 and SEBTA-005) (Figure 39 C). The level of CD15 expression was higher in SEBTA 001 and SEBTA-005 which agree with the ICC results. Flow cytometry analysis indicated 2% of SC-1800 cells were positive for CD15s compared to isotype controls. 8% of SNB-19 cells and 16% of UP-007 were CD15s positive in non-synchronised glioma cells whereas in lung to brain metastatic cell lines expressed 34% (SEBTA-005) and 47% (SEBTA-001) (Figure 39 D and E). Both the lung to brain metastatic cell lines were significantly higher in terms of their CD15s positive cells compared to isotype controls (p<0.001) and the glioma cell lines (p<0.01).
Figure 39. Expression and localization of CD15s in non-synchronised cells. A: Immunocytochemistry images showing no CD15s expression (green) in non-neoplastic astrocytes (SC-1800) but positivity in in primary GBM cells (UP-007 and SNB-19) and secondary brain cancer cells (SEBTA-001 and SEBTA-005). Hoechst blue was used as a nuclear counterstain. Scale bar =20μm. B: Semi-quantification analysis of CD15s from confocal microscopy images using Zeiss Zen image software. C: Western blot analysis showing no CD15s expression in human astrocytes, weak expression in GBMs (UP-007 and SNB-19) and highest expression in brain secondary metastatic cancer cells (SEBTA-001 and SEBTA-005). D: Representative flow cytometric histograms and E: Quantitative flow cytometric analysis of CD15s expression in SC-1800, UP-007, SNB-19, SEBTA-001 and SEBTA-005. CD15s was highly expressed in SEBTA-001 and SEBTA-005 and least expressed in SC-1800. *(P≤0.05), ** (P<0.01) and *** (P<0.001) difference compared to the IgM isotype control. Results are representative of three independent experiments carried out in triplicate (n=3).
5.4 Synchronisation of cell cultures

All of the cell cultures were synchronised to G1, S and G2/M phase of the cell cycle using serum depletion, hydroxyurea and Nocodazole respectively. Efficiency of synchronisation was determined in each in cell line using DNA analysis via flow cytometry (Figure 40). Results of flow cytometry analysis of propidium iodide stained cells from serum depletion showed most cells were arrested at G1 phase at 94% in SC-1800, 91% in UP-007, 92% in SNB-19, 92% in SEBTA-001 and 90% in SEBTA-005 (Figure 40). Hydroxyurea blocks the *de novo* synthesis of DNA through inhibiting the activity of ribonucleotide reductase. Results showed that where cell cultures were incubated at 1mM/mL of Hydroxyurea, 55% of SC-1800 cells, 42% of UP-007, 48% of SNB-19, 64% of SEBTA-001 and 53% of SEBTA-005 were synchronised in S phase; the slight discrepancy in synchronisation of astrocytes is considered acceptable due to the unbalanced cell growth caused by the treatment with hydroxyurea which is an anti-cancer drug as well as the high heterogeneity of cancer cell cultures which caused a different response to hydroxyurea among individual cells. The short period of transition from G phase to G2/M phase also made it unlikely that there would be a high number of cells arrested at S phase (Figure 40). The third arrest point was G2/M phase. Cells were induced to enter G1 phase then treated with 2μg/mL Nocodazole as a mitotic inhibitor for 24 hours. Results showed that 61% of SC-1800 cells, 82% of UP-007, 53% of SNB-19, 68% of SEBTA-001 and 61% of SEBTA-005 were arrested at G2/M phase (Figure 40).
Synchronisation of cell cultures. Cell cultures were synchronised at different cell cycle stages using serum depletion to arrest cells at G1 phase, 1mM/L hydroxyurea for S phase and 2µg/mL Nocodazole for G2/M phase. Flow cytometry analysis was used to assess the efficiency of cell synchronisation and the distribution of arrested cells in each phase. 91% of SC-1800, 91% of UP-007, 92% of SNB-19, 92% of SEBTA-001 and 90% of SEBTA-005 were arrested in G0/G1 phase after 48 hours of serum depletion treatment. 55% of SC-1800, 42% of UP-007, 48% of SNB-19, 64% of SEBTA-001 and 53% of SEBTA-005 cells were synchronised in S phase by incubation in 1mM/L for 24 hours in full growth medium. 61% of SC-1800, 82% of UP-007, 92% of SNB-19, 92% of SEBTA-001 and 90% of SEBTA-005 of cells were arrested in G2/M phase after treatment with 2µg/mL Nocodazole for 24 hours.
5.6 Expression of CD15 at different cell cycle stages

Two methods were used to determine if CD15 is expressed in a cell cycle dependant manner with one synchronised as described above and a qualitative method using a Premo™ (FUCCI) cell cycle sensor. In synchronised cells, flow cytometry analysis revealed that CD15 was expressed at low levels in SC-1800 with no observable difference in expression in G1 (1.8%), S (1.18%) or G2/M (1%) whilst in GBM cells, CD15 expression was significantly higher in cells arrested at G1 phase: 14.7% and 21.4% in UP-007 (p<0.05) and SNB-19 (p<0.01) respectively compared to non-synchronised counterparts (Figure 5 A and B). In addition, in each of the GBM cell lines there was significantly higher CD15 expression in G1 compared to S (p<0.01) G2/M (p<0.01). Similarly CD15 was significantly elevated in secondary brain tumour cells synchronised in G1 phase (p<0.01) with positivity of 66% in SEBTA-001 and 38% in SEBTA-005 compared to non-synchronised cells (Figure 41 A and B). These data were supported by qualitative results obtained from transfected cells using the Premo™ (FUCCI) cell cycle sensor to differentiate specific cell cycle stages, visualised using a LSM 510 Axioskop2 confocal microscope. G1, S and G2/M phases were detected by red, yellow and green nuclei respectively. In these non-synchronised studies, CD15 cell surface expression (green) was mostly co-localised with red nuclei indicative of G1 (Figure 41C) supporting that higher levels of CD15 expression can be detected in cells at G1 phase of the cell cycle.
Figure 41. Expression and localisation of CD15 during different stages of the cell cycle. A: Flow cytometric histograms representing CD15 expression in synchronised cells SC-1800 (1), UP-007 (2), SNB-19 (3), SEBTA-001(4) and SEBTA-005(5) cells. CD15 positive expression is represented by a shift to the right. B: Statistical analysis of flow cytometry results showing that the highest level of CD15 expression was detected in G0/G1 synchronised cells followed by non-synchronised cells and G2/M phase. Cells are arrested at S phase showed that the least CD15 expression level. *(P≤0.05), ** (P<0.01) and *** (P<0.001) difference compared to the IgM isotype control. C: Immunocytochemistry results of CD15 extracellular expression (green). Cells were treated with FUCCI™ system to differentiate specific cell cycle stages depending on the colour of the nuclei (red: G1; yellow: S and green: G2/M phase). Results show CD15 expression was detected at low levels on SC-1800 cell with red nuclei (G1 phase). In GBM cells (UP-007 & SNB-19) and metastatic lung-brain cancer cells (SEBTA-001 & SEBTA-005), CD15 was expressed and well distributed on cell surfaces with red or yellowish-red nuclei (arrow) indicating G1 phase. Scale bar is 20μm. Results are representative of three independent experiments carried out in triplicate (n=3).
5.7 Expression of CD15s at different cell cycle stages

CD15s expression also appears to be cell-cycle dependent. As with CD15, CD15s expression in the astrocyte cell line was low and did not significantly change with synchronisation. In GBM cells, CD15s expression was significantly higher in cells arrested at G1 phase: 39% and 48% in UP-007 (p<0.001) and SNB-19 (p<0.001) respectively compared to non-synchronised counterparts (Figure 42 A and B). In addition, in each of the GBM cell lines there was significantly higher CD15s expression in G1 compared to S (p<0.001) G2/M (p<0.001). Similarly CD15s were elevated in secondary brain tumour cells synchronised in G1 phase (p<0.05) with positivity of 44% in SEBTA-001 and 27% in SEBTA-005 compared to non-synchronised cells (Figure 42 A and B). In non-synchronised co-localisation studies, CD15s expression (green) was also co-localised with red nuclei indicative of G1 (Figure 42 C). These results suggest that over-expression of CD15s correlates with G1 phase.
Figure 42. Expression and localisation of CD15s during different stages of the cell cycle.

A: Immunocytochemistry results of CD15s extracellular expression (green). Cells were treated with the FUCCI™ system to differentiate specific cell cycle stages depending on the colour of the nuclei (red: G1; yellow: S and green: G2/M phase). Results showed that CD15s expression was detected at low level on SC-1800 cells with red nuclei (G1 phase). In GBM cells (UP-007 & SNB-19) and metastatic lung-brain cancer cells (SEBTA-001 & SEBTA-005), CD15s was expressed and well distributed on cell surfaces with red or yellowish-red nuclei (arrow) indicating G1 phase. Scale bar=20μm. B: Flow cytometric histograms representing CD15s expression in synchronised cells: SC-1800 (1), UP-007 (2), SNB-19 (3), SEBTA-001(4) and SEBTA-005(5) cells. CD15s positive expression is represented by a shift to the right. C: Statistical analysis of flow cytometry results show that the highest level of CD15s expression was detected in G0/G1 synchronised cells followed by non-synchronised cells and G2/M phase. Cells which arrested at S phase showed lowest CD15s expression levels. *(P≤0.05), ** (P<0.01) and *** (P<0.001) difference compared to the IgM isotype control. Results are representative of three independent experiments carried out in triplicate (n=3).
5.8 Discussion

CD15 and CD15s are fucosylated polysaccharide epitopes and tumour-associated cell adhesion molecules. Overexpression of both epitopes has been correlated with malignancy of many non-CNS cancers (Ohana-Malka et al., 2003). Early studies in CNS cancers reported the absence or low expression in GBM cells (Mai JK, Reifenberger 1988; Martin et al., 1995). However more recently, overexpression of CD15 in high grade malignant CNS tumours has been reported (Mao et al., 2009 and Kahlert et al., 2012). In addition, CD15 has been documented as a marker of self-renewing stem-like cells in GBM (Kahlert et al., 2012). In contrast, Kenney-Herbert et al, (2015) reported that there were no phenotypic or genetic differences between CD15- and CD15+ GBM cells and CD15 expression was not enough to distinguish a discrete population of GBM cells. Few studies have however, considered the possibility of cell-cycle dependent CD15 expression which may explain the inconsistencies reported in the literature. CD15s has also been associated in malignancy of many non-CNS cancers (Ogawa et al., 1994 and Sozzani et al., 2008). CD15s has frequently been reported to correlate with invasiveness of cancer cells, for example in head and neck carcinoma where it was also proposed as a cancer stem cell marker (Czerwinski et al., 2013). In GBM, CD15s expression has also been shown to be highly heterogeneous within different non-synchronised cell lines of GBM with positivity roughly from 4.8%-32.8% (Lou et al., 2015) which is in agreement with our findings that show the variability in expression in non-synchronised GBM cell lines (UP-007 and SNB-19). In non-neoplastic astrocytes, CD15 and CD15s expression was low and this did not change when cell lines were synchronised to G1, S, or G2/M consistent with reports showing low CD15 expression in astrocytes (Martin et al., 1995). In the GBM cell lines tested the expression of CD15 and CD15s was significantly higher when cells are synchronised in G1 phase. In secondary lung-brain tumour cells, higher levels of CD15 were seen in non-synchronised cells which perhaps relates to the fact that CD15 is well known to be elevated in lung
carcinomas (Kadota et al., 1999). These findings suggest that overexpression of both CD15 and CD15s in GBM cells and secondary lung-brain tumour cell correlate with G1 phase. Our overall findings suggest that in GBM cell lines, CD15 and CD15s expression is correlated with specific stages of the cell cycle, in particular G1. This may help explain the opposing reports in the literature concerning CD15 expression in glioma cells and ‘glioma stem-like cells’ as it could be that due to ‘stem-like cells’ spending more time in the S phase where CD15 and CD15s expression would be low. Whether this is the case remains to be determined and requires additional research. An additional interesting question is do CD15 and CD15s play a role during cell cycle progression, particularly while cells are in G1. It has been suggested since the late 1980’s that cells in G1 were more susceptible to initiate differentiation in response to growth factors (Mummery CL et al). The concept of connecting the cell cycle phase and cell fate as well as the time spent in each phase has gained supportive experimental evidence over the years (reviewed in Dalton, S Trends in cell biology 2015; Hardwick and Philpott Trends in genetics 2014) Recently Singh (2015) proposed a hypothetical model of the relationship between cell cycle, pluripotent cells and heterogeneity and that G1 could serve as a ‘differentiation induction point’ (Singh, AM 2015 Stem cells international). Could, as proposed by Hardwick and Philpott (2014), the length of time ‘glioma stem-like cells’ are in G1 be targeted to enhance differentiation and slow tumour progression? How does the glioma ‘cancer stem cell niche’ contribute to the cell cycle length? In an interesting proteomic study using a breast cancer cell line in G1, bioinformatic tools and databases, Tenga and Lazar (2014 – BMC) reported from their study that three major clusters of interacting networks emerged and included oxidative phosphorylation, DNA repair and signalling. Instead of relying on ‘glioma stem-like cell’ markers the idea of using molecular regulatory components that act within a network may prove promising in terms of understanding and identification of therapeutic targets for GBM and ‘stem-like cells’.
Expression of CD15 and CD15s in lung to brain metastatic cell lines is also correlated with the G1 phase. We have recently shown that CD15 in lung to brain metastasis plays a role in binding to brain endothelial cells \(^{17}\) and our current data helps direct future investigations to address the question of how circulating lung cancer cells and their cell cycle stage influence adherence to endothelial cells of blood capillaries during brain metastasis. This may be critical in terms of determining preventative therapeutics for brain metastasis as many drugs arrest cells in specific cell cycle phases. For example, a drug causing cell cycle arrest at G1 may not be recommended if CD15 and CD15s are highly expressed in this phase as it would result in an increase in adhesion to brain endothelial cells. Although highly speculative, this points for the need for further research into this area.
CHAPTER SIX

CONCLUSIONS
CD15 and CD15s are overexpressed in metastatic lung cancer cells. CD62E is expressed on brain endothelium stimulated with TNF-α and interaction between CD15/CD15s and CD62E plays an important role in lung cancer cell adhesion to brain endothelium cells.

CD15 and CD15s are important cell-cell adhesion molecules and are both implicated in metastasis in many non-CNS malignancies (Kadota et al., 1999 and Giordano et al., 2015). The exact functional role of these molecules in metastasis to the CNS is not known. In this project, expression of CD15 was characterised in a range of primary and metastatic human NSCLC cells. ICC images showed a prominent and condensed expression of CD15 on the outer edges of cell surface of metastatic lung cancer cells compared to less expression on the primary lung cancer cells. These results were confirmed by Western blotting and flow cytometry analysis (Figure 19). CD62E is an important ligand of CD15 and CD15s.

Adhesion of lymphocytes to endothelial cells has been shown to be regulated by the heterophilic interaction between these two molecules (Homeister et al., 2001). We hypothesised that metastatic lung cancer cells may mimic lymphocyte behaviour and investigated CD62E expression in brain endothelial cells after 18 hours stimulation with TNF-α (25pg/mL). Results demonstrated that CD62E was highly expressed in human brain endothelial cells. Stimulation with TNF-β was also used to confirm the specificity of TNF-α (Figures 22 and 23). We then investigated the role of CD62E and CD15 in adhesion of lung cancer to brain endothelial cells. Results of qualitative and quantitative adhesion assays revealed that CD62E play a key role in the adhesion process of cancer cells and its absence significantly reduced the number of adherent cells (Figure 24). Findings also showed that metastatic lung cancer cells were more adherent than primary lung cancer cells and immunoblocking of CD15 significantly reduced the adhesion of cancer cells (Figure 24). These results suggested a strong correlation between CD15 and the adhesion of lung cancer cells on brain endothelium. Similarly, immunoblocking of CD15 reduced
the adhesion of highly metastatic lung to brain cancer cells in a dynamic flow experiment which was conducted under shear stress at perfusion rate of 2.5 dyn/cm² (Figure 24). CD15-CD15 homophilic interactions were localised between adjacent cancer cells (Figure 25). In addition, the heterophilic binding between CD15 and CD62E was localised at the site of adhesion of metastatic lung cancer cells adhering on brain endothelial monolayer (Figure 25C) and this co-localisation was confirmed by 3-dimantional confocal imaging generated by Z-stack (Figure 25D).

CD15s was seen to be expressed at low levels on the cell surface of brain endothelium and primary NSCLC and a higher level on metastatic lung cancer cells. It was found also to be expressed in human tissue sections of brain tumour metastasised from lung while no CD15s was seen in tissue sections of normal brain (Figure 27). A qualitative adhesion assay showed that immunoblocking with CD15s monoclonal antibodies significantly reduced the number of adherent cancer cells on activated monolayer of brain endothelial cells. These findings were assessed by quantitative adhesion assay which showed a noticeable decrease in adhesion ability of cancer cells following immunoblocking with CD15s antibodies under static conditions (Figure 28 and 29). We then examined whether CD15s may play a role in cancer cell adhesion to brain endothelial monolayer under shear stress with flow rate of 2.5 dyn/cm². Findings showed that blocking with CD15s monoclonal antibodies reduced the adhesion ability of highly metastatic and adherent lung-brain metastatic cells (SEBTA-001) (Figure 30). CD15s expression was also seen to be localised on the surface of adherent cancer cells with prominent staining on the edges of the cancer cells boundary at the site of the adhesion (Figure 31). Both CD15 and CD15s were seen to be expressed in human tissue section of brain metastatic brain tumour originated from lung, however, CD15 and CD15s were not seen in normal brain tissue as in figure (26 and 32).
Knockdown of FUT4/CD15 and FUT7/CD15s reduced the transmigration potential of cancer cells and overexpression of FUT4/CD15 and FUT7/CD15s accelerated the transmigration of cancer cells through a brain endothelial monolayer.

The haematogenous pathway is the major route in brain metastasis and circulating cancer cells usually reach the brain through transmigration across BBB capillaries. Recently, the substantial role of brain endothelial in brain metastasis was highlighted. Overexpression of CD62E on brain endothelium was detected at the early stages of tumour seeding (Soto et al., 2014). We investigated the role of CD15 and CD15s in adhesion of cancer cells to brain microvascular endothelial cells (hCMEC/D3) as well as their role in transendothelial migration across the endothelial monolayer where a previous study showed that CD15 and CD15s played a role in the leukocytes homing process (Strell and Entschladen, 2008). The functional role of these molecules in metastasis to the CNS is still not fully explained. Here, we investigated the effect of knockdown and overexpression of FUT4/CD15 and FUT7/CD15s in adhesion of NSCLC to brain endothelium as well as their role in transmigration across a brain endothelial monolayer. Our findings showed that knockdown of fucosyltransferase IV (FUT4) reduced the expression of CD15 whereas its upregulation led to CD15 overexpression in GBM, lung primary and metastatic cancer cells (Figure 32). FUT7 knockdown significantly reduced the expression of CD15s while upregulation of FUT7 induced the overexpression of CD15s (Figure 33). We then examined the effect of FUT4/CD15 and FUT7/CD15s genetic manipulation on the adhesion of transfected cells and findings showed that FUT4/CD15 knockdown decreased the adhesion ability of cancer cells while FUT4/CD15 increased adhesion in all studied cancer cells (Figure 34A). Similarly, we found that adhesion was significantly reduced by knockdown of FUT7/CD15s and notable increase in cancer cell adhesion was induced by upregulating FUT7/CD15s (Figure 34B). We also investigated the transendothelial migration potential of above mentioned cancer cells using three different approaches (EVOM, ECIS and
CellZscope). This was to validate our results and show consistency in our findings irrespective of equipment used. Results revealed that the highest transendothelial migration was seen in metastatic lung cancer cells: SEBTA-001 and NCI-H1299. Both cell lines caused a significant and accelerated decrease in TEER value in the brain endothelial monolayer unlike the primary lung cancer cells COR-L105 which caused a small and non-significant decrease in TEER value and GBM cells hardly caused any decrease in TEER in the endothelial monolayer (Figure 35 A,B and C). In transfected cells, results showed that silencing FUT4/CD15 decreased transendothelial migration of highly metastatic as seen by a decrease in TEER values. Overexpression of FUT4/CD15 increased cancer cells transmigration across the endothelial monolayer suggesting that FUT4/CD15 plays a role in transendothelial migration of cancer cells during metastasis to the brain (Figure 36 A,B and C). FUT7/CD15s silencing clearly reduced cancer cell to transmigrate via the brain endothelial monolayer as demonstrated by no decrease in the resistance of the endothelial monolayer (TEER) value. These findings suggest that FUT4/CD15 and FUT7/CD15s play a major role in transendothelial migration during NSCLC seeding to the brain.

**CD15 and CD15s expression was correlated with cell arrest at G1 phase in primary and metastatic brain cancer cells.**

CD15 and CD15s have both been reported to be correlated with malignancy and progression to the metastatic stage in many non-CNS neoplasms (Gadhoum et al., 2008, Rho et al., 2014, Fukusumi et al., 2014 and Desiderio et al., 2015). Knowledge regarding CD15 expression in CNS malignancies however is limited and even less is known about the expression of CD15s. In order to understand the discrepancy in literature regarding CD15 and CD15s expression and distribution in CNS neoplasms, we characterised CD15 and CD15s in non-neoplastic adult astrocytes, a range of human primary brain tumour (GBM) cells and secondary brain tumour cells that had metastasised from lung during
different stages of cell cycle for CD15 and CD15s expression. Our findings showed that in non-synchronised cells, CD15 was hardly seen in adult astrocytes and only expressed at low levels in GBM cells (5%-15%) whereas a prominent expression of CD15 was seen in metastatic brain tumour cells (28%-36%) (Figure 38). CD15s was expressed in 2% of non-neoplastic astrocytes while 8%-26% of primary brain cancer cells expressed CD15s and higher levels of expression were seen in secondary brain tumours with positivity of 34%-47% (Figure 39). Synchronisation of cultures was done: G0/G1 phase by serum starvation, S phase by hydroxyurea (1mM) via inhibition of ribonucleotide reductase activity (Maurer-Schultze et al., 1988) and G2/M phase by Nocodazole (2μg/mL). It inhibits the polymerisation of microtubule (Matsui et al., 2012 and Rosner et al., 2013) (Figure 40). Flow cytometry was used to assess the synchronisation in treated cells followed by CD15 and CD15s expression localised and measured using a fluorescent cell cycle indicator system (FUCCI) and flow cytometry analysis respectively. In synchronised cells, FUCCI results showed that CD15 was seen to be expressed in cells with red and red-yellowish nuclei in non-neoplastic astrocytes, primary and secondary brain tumour cells. These findings indicated that CD15 was mostly expressed on cells arrested at G1 phase. Flow cytometry results validated FUCCI findings and highest level of CD15 expression was seen in cells which were synchronised at G1 phase (Figure 41). CD15s was expressed on the surface of cells at G1 phase in non-neoplastic astrocytes, primary brain tumour cells and secondary brain tumour cells. Faint expression of CD15s was also seen on cells at S and G2/M phase. Flow cytometry analysis showed that the highest levels of CD15s expression was detected in cells synchronised at G1 phase in non-neoplastic astrocytes, primary and secondary brain tumour cells (Figure 42).
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APPENDICES
Appendix 1: DNA profiling of studied cells (cell authentication)

A  hCMEC/D3  SC-1800
SNB-19  UP-007
A549  COR-L105
SEBTA-001  SEBTA-005
NCI-H1299
### B- STR genotypes of the cell lines

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Appendix 1: DNA profiling of studied cells conducted by microfluidic system (Agilent 2100 Bioanalyzer). A: Representative single electropherograms of short tandem repeat (STR) profiles of the cell lines: hCMEC/D3, SC-1800, SNB-19, UP-007, 549, A549, COR-L105, SEBTA-001, SEBTA-005 and NCI-H1299. Samples were analysed based on the molecular size as recorded above each peak B: A unique DNA profile of each cell line shown by fragment sizing. The electropherograms were illustrated via Agilent 2100 Expert software and experiments were conducted in triplicates for three independent times. (Acknowledgement: Mrs. Katie Loveson).
Appendix 2: Cellular growth curves of cell lines used throughout the project. Cell count was determined using a VICELL XR machine. Measurements were conducted in triplicate in three independent experiments.
Appendix 3A: Cloning scheme for RG223829. *FUT4*- human cDNA ORF clone pCMV6-AC-GFP retroviral GFP vector ready-cloned, contains unique construct of alpha (1,3) fucosyltransferase, myeloid-specific protein (FUT4), CMV promoter, T7 promoter, G418 resistance gene, C-terminal TurboGFP tag (Obtained from Origen).

Appendix 3B: Cloning scheme for RG223829. *FUT7*- human cDNA ORF clone : pCMV6-AC-GFP retroviral GFP vector ready-cloned, contains unique construct of alpha (1,3) fucosyltransferase (FUT7) protein, CMV promoter, T7 promoter, G418 resistance gene, C-terminal TurboGFP tag (Obtained from Origen).
# Introduction

**Title of Thesis:** Role of CD15 and CD15s in metastasis from lung to the brain

**Thesis Word Count:** less than 80,000 words

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**UKRIQ Finished Research Checklist:**

- a) Have all of your research and findings been reported accurately, honestly and within a reasonable timeframe? **YES**
- b) Have all contributions to knowledge been acknowledged? **NO**
- c) Have you complied with all agreements relating to intellectual property, publication and authorship? **NO**
- d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration? **NO**
- e) Does your research comply with all legal, ethical, and contractual requirements? **NO**

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**Candidate Statement:**

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s).

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC): 11/SC/0048

If you have **not** submitted your work for ethical review, and/or you have answered ‘No’ to one or more of questions a) to e), please explain below why this is so:

---

Signed (PGRS): [Signature]

Date: [Date]

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**Postgraduate Research Student (PGRS) Information**

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**Study Mode and Route:**

- [ ] Part-time
- [ ] Full-time
- [ ] MPhil
- [ ] MD
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- [ ] Professional Doctorate

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