ARTERIOVENOUS MALFORMATION ENDOTHELIUM AND RESPONSES TO RADIATION

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This thesis is dedicated to my parents, Amal and Suja.

Declaration of originality

I certify that the research described in this thesis has not been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged.

Ethics Committee approvals for all patient data collected were obtained from Macquarie University Human Ethics Committee (Number: 5201100266). Ethics Committee approvals for all animal work were obtained from Macquarie University Animal Care and Ethics Committee (Number: 2012029 and 2014051).

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Table of Contents

List of Figures and Tables

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Abbreviations

ACD	Acid citrate dextrose
AVM	Arteriovenous malformation
BMP	Bone morphogenetic protein
CCA	Common carotid artery
CD51	Integrin V alpha
CD105	Endoglin
CD109	TGF binding protein 1
EC	Endothelial cells
EF2	Elongation factor 2
EJV	External jugular vein
ELISA	Enzyme linked immunosorbent assay
EMP	Endothelial microparticle
ESAM	Endothelial cell adhesion molecule
FS	Forward scatter
GKS	GammaKnife Surgery
HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase
HHT	Hereditary haemorrhagic telangiectasia
iTRAQ	Isobaric tag for relative and absolute quantitation
LC	Liquid Chromatography
LINAC	Linear accelerator
MS	Mass spectrometry
PET	Positron emission tomography
NO	Nitric oxide
NOS	Nitric oxide synthase
PECAM	Platelet endothelial cell adhesion molecule
SAGE	Serial analysis of gene expression
SEM	Scanning electron microscopy
STAT	Signal transducers and activators of transcription
TGF	Transforming growth factor
VCAM	Vascular cell adhesion molecule

VEGF	Vascular endothelial growth factor
VDA	Vascular disrupting agents
VT	Vascular targeting
VTA	Vascular targeting agents

Abstract

Cerebral arteriovenous malformations (AVMs) are congenital lesions that cause brain haemorrhage leading to death or permanent neurological disability in children and young adults. Treatment options to prevent haemorrhage include surgery, radiosurgery and embolisation. Depending on the size and location of the lesion, over one-third of AVMs cannot be treated safely with current options. Therefore, there is a need for a new treatment.

It is proposed that vascular targeting could be a novel effective therapy for AVMs that are currently untreatable. An antibody attached to an effector molecule such as thrombin or tissue factor may be targeted at a specific molecule expressed in AVM vessels to create intravascular thrombosis. A better understanding of the AVM endothelial molecular biology is thus required for identifying the best candidates for vascular targeting in AVMs.

Vascular targeting has been applied successfully in the treatment of cancer because of the significant differences between the tumour and normal vasculatures. This however is not the case in AVMs. It is proposed that radiosurgery can 'prime' AVM endothelial cells by stimulating them to express certain proteins, which could then be targeted.

This thesis aims at identifying molecular changes in the endothelial cells exposed to irradiation for the development of a potential vascular target. To accomplish this, radiation-induced molecular changes were investigated in human brain microvascular endothelial cells as well as in animal models of AVM. Furthermore, endothelial microparticles were characterised to evaluate molecular changes in human AVMs.

Previous *in vitro* studies on mouse brain endothelial cells have shown that irradiated cells express CD51, CD109 and BMP3 protein markers. One of the aims of this project was to determine the localisation of these three molecules in the endothelium of AVM animal models that were exposed to irradiation and also

to identify the proteins expressed in human cerebral microvascular endothelial cells in response to radiation.

Studies in rat models of AVM showed increased abundance of BMP3 in AVM vessels on day 21 post-irradiation. *In vitro* studies showed talin to have increased relative abundance in LC/MS/MS analysis, increased band intensity in western blot analysis and increased fluorescent intensity in immunofluorescence analysis. These results add to our understanding of AVM endothelial responses to radiation. BMP3 and talin are promising protein markers for AVM vascular targeting. Further work is required to determine whether these are clinically viable and effective targets.

Chapter 1

Introduction

Arteriovenous Malformations and Endothelial Cells

Definition

"Arteriovenous malformations (AVMs) of the brain are focal abnormal conglomerations of dilated arteries and veins within brain parenchyma, in which a loss of normal vasculature organisation at the sub arteriolar level and a lack of a capillary bed result in abnormal arteriovenous shunting" [1].

Introduction

AVMs are generally thought to be congenital although there is some evidence to suggest that they form post–natally [2]. They can occur in any part of the central nervous system and are the most common cause of intracerebral haemorrhage in children and young adults [3]. Arteries and veins are connected by one or more fistulae, which form a tangled structure commonly known as the AVM '*nidus*' (Fig. 1.1). These direct connections allow high-pressure arterial blood to flow through the fragile cerebral veins, which carry a high risk of rupture with ruinous results [1]. Factors that are associated with a higher risk of haemorrhage include small size, location and shunt resistance of the AVM [4]. Other characteristics include the presence of aneurysms, a single draining vein and venous stenosis [1].

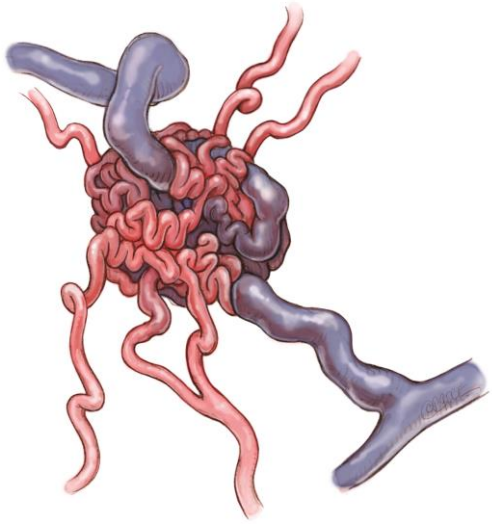

Figure 1.1: Arteriovenous malformations
AVMs are short circuit connections between arteries and veins that form a tangled web called a '*nidus*'. (Figure - courtesy of Prof. Stoodley)

Symptoms

The most frequent presentation of brain AVMs is intracranial haemorrhage [5]. Over 50% of 1289 AVM patients presented with haemorrhage [6]. This was followed by epileptic seizures in 25%, chronic headaches in 15% and progressive neurological deficits in 5% [6]. There is a 30% risk of permanent neurological disability and a 15% risk of death in every incidence of bleeding [7]. The level of neurological deficit increases with the depth of the *nidus* [8].

Pathophysiology

AVMs are considered to be congenital lesions that are the result of disordered development of the primordial vasculature. This hypothesis is criticised by some due to the absence of strong evidence in support of the theory. AVM patients are usually 20 to 60 years of age at presentation [9]. AVMs occur sporadically and are not associated with non central nervous system congenital malformations [9]. Some studies refer this 'puzzle' to teratologists so that a better understanding of AVMs could be achieved [3, 9].

In a study that involved over 1500 AVM cases, it was concluded that a biological change occurs only around 10 years of age [10]. Before that, the AVM could have been absent totally or in an inactive state [10]. There are also studies that report the regrowth of AVMs after successful treatment [3]. The above provide evidence against the traditional assumption that AVMs are of congenital origin.

Future studies are needed to explore the pathogenesis of AVMs [3]. Research on the molecular biology of brain AVMs may one day be able to prove that AVMs are acquired abnormalities that develop in the postnatal period.

Epidemiology

Studies show the incidence of AVM detection in various populations to be 0.9/100000 to 1.4/100000 per year. The prevalence of AVMs is estimated to be 0.01% of the population [11].

The incidence of AVMs is fairly low when compared with most other neurologic disorders such as stroke, epilepsy, intracranial haemorrhage, benign central nervous system tumour, motor neuron disease and spinal cord injury [12]. The

average age of AVM presentation is mid-30s. Various population-based and large natural history studies have shown that there is no gender predilection in AVMs [13].

Population based studies show a high incidence rate in Kaiser Permanente (1.4/100,000) and in the New York Islands (1.3/100,000). This is followed by Sweden (1.2/100,000), The Netherlands Antilles and Olmstead County, Minnesota (1.1/100,000) and Western Australia (0.9/100,000) [13].

Studies show that 2% of AVM cases are associated with Hereditary Haemorrhagic Telangiectasia (HHT), an autosomal vascular disorder [14]. The incidence of HHT associated AVMs is 1 in 5000-8000 individuals which can get much higher in the Dutch Antilles (1 in 1300) [15].

Classification of AVMs

The Spetzler-Martin grading system is currently used to predict the risk of surgery for the AVM patient. According to that system, AVMs are classified based on their size, pattern of venous drainage and neurological eloquence of the brain regions adjacent to them [16]. This is a more practical approach than considering all the other factors associated in the risk prediction process such as number of feeding arteries, amount of flow through the lesion, degree of steal from surrounding normal brain, location, surgical accessibility, eloquence of adjacent brain and pattern of venous damage [17].

Angiography, computerised tomography (CT) and magnetic resonance imaging (MRI) are currently used for the grading of AVMs based on the three categories mentioned above. Each of the categories is given a numerical value and the values are summed to derive the grade of the lesion. The lesions are graded from I-V, where I is the least and V is the maximum in terms of risk of surgical morbidity and mortality. Additionally, there are grade VI AVMs that are inoperable because of their large size and critical location in the brain region [16].

Treatment options

At present, AVMs can be treated by surgery, endovascular treatment or radiosurgery, all of which aim at preventing a haemorrhage. The treatment option is chosen depending on various factors such as size, location and anatomy of the AVM and previous history of haemorrhage.

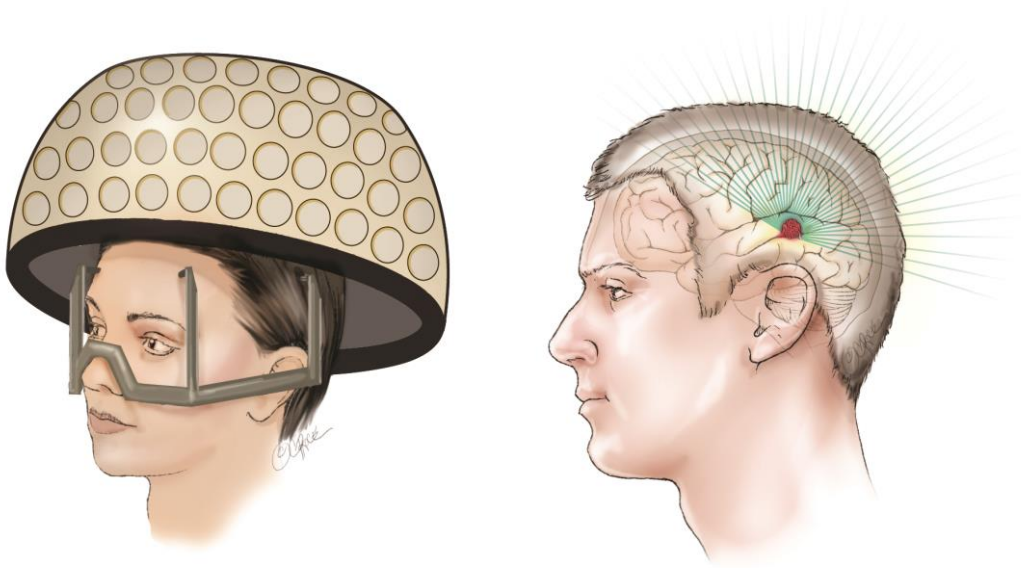

Figure 1.2: Radiosurgery
Radiosurgery is a non-invasive mode of treatment where focussed irradiation is administered to the AVM without any effect on the surrounding tissues.
(Figure - courtesy of Prof. Stoodley)

Currently, surgical resection is the best treatment for AVMs because it provides immediate cure [18] [19]. This is achieved by the removal of the nidus along with the direct feeders and disconnection of the draining vein [18]. Factors such as size and location of the lesion are taken into consideration by the treating neurosurgeon prior to surgery [16]. Not all AVMs can be treated by surgery [20]. This mode of treatment can produce high cure rates without further complications only for Grade I, II and most Grade III AVMs [21]. The effectiveness of surgery in such AVMs has been confirmed in a study where the AVM was successfully obliterated from 99% of 110 patients with Grade I-III AVMs. Grade IV and V AVMs carry a high surgical risk if they are to be operated and Grade VI AVMs are inoperable [21].

In stereotactic radiosurgery, a dose of focussed radiation is accurately administered to the AVM nidus (Fig. 1.2) [22]. This may be achieved using

Gamma Knife, linear accelerators and proton beam [22]. Radiosurgery is an ideal method for treating AVMs that cannot be resected due to their critical locations in the brain [21, 23]. The success rate is high in AVMs that are <2.5 cms in diameter. Studies show a 60 – 80% chance of obliterating the AVM successfully [21]. Despite its advantages of being less invasive and cost effective when compared to surgical resection, radiosurgery poses its own limitations [23]. There is a latent period of 3 years during which a haemorrhage could possibly occur [18]. This has been observed in a study where 4% of 602 patients suffered from intracranial haemorrhage during the latency period [23]. In some cases, the AVM could be unresponsive to the treatment even after the 3 years due to factors such as radiobiological resistance, nidus size, number of draining veins, deep location and patient age [18].

Endovascular embolisation is another possible treatment for brain AVMs. This is popular mainly because of its benefits of being minimally invasive, quick in occluding the high-pressure shunt of blood and intraprocedure angiographic evaluation. Embolisation is carried out exploiting advances in endovascular technologies where unique liquid embolics are developed and delivered through a micro catheter. Ethylene vinyl alcohol copolymer, n-butyl cyanoacrylate and platinum embolic coils are the commonly used liquid embolics. This treatment may be carried out prior to surgery or in conjunction with radiosurgery. Large AVMs may be reduced in size through embolisation for further treatment by surgery or radiosurgery. Endovascular therapy however is effective only for small AVMs. Other limitations include incomplete embolisation, intracranial haemorrhage, unintended vessel embolisation and normal perfusion pressure breakthrough leading to swelling or haemorrhage [24].

The above treatments, despite their benefits can only treat small AVMs effectively. Over 90% of the grade IV and V AVMs remain untreatable and 25% of the grade III AVMs cannot be treated without high risk [16, 25]. There is therefore an urgent need for a new treatment option to save those patients with large AVMs that cannot be treated and run the risk of a haemorrhage or sudden death [26].

Potential new treatment for AVMs

Theoretical changes to the current, existing treatment options are very unlikely to happen for the successful treatment of AVMS that are large and those in critical locations. We are therefore counting on novel molecular and cellular based therapies such as vascular targeting, vascular remodelling and radiosensitisation for the treatment of AVMs [27-29].

Over 900 genes encode for growth factors, extracellular matrix, matrix metalloproteinase, endocrine hormones and inflammatory factors and are either up-regulated or down-regulated in brain AVMs [29-33]. This opens the way for possible gene therapies as potential new treatments. Endothelial cells, vascular smooth muscle cells and inflammatory cells are being studied to add to the limited knowledge we have on the pathogenesis and molecular biology of AVMs [33]. In molecular studies, integrins, angiotensins, VEGF, TGFβ have been shown to play a critical role in the pathogenesis of AVMs [33]. Although informative regarding AVM pathogenesis, these molecular findings are unlikely to be useful for treatment once an AVM has already formed.

A new treatment is proposed to induce thrombosis in the AVM vessels without any effect on the surrounding vessels. The aim is to block the high-pressure arteriovenous shunt. For this to be successful, it is required to identify a molecule that occurs on the internal surface of the blood vessels of AVMs specifically and not in the surrounding vessels. Once identified, that signature molecule located on the internal surface of the blood vessels can be targeted with an antibody. The antibody will be attached to another molecule that will stimulate thrombosis. It is hoped that when the therapy is injected systemically, it will adhere to only the AVM blood vessels and promote thrombosis in the AVM. In order to do that, it is needed to identify a signature molecule on the endothelial surface. It might not be possible to identify one that is specific enough in AVMs to be able to use that in AVM treatments. Therefore radiosurgery is proposed to stimulate that signature. Radiation will stimulate molecular changes in the AVM tissues, but in particular, in the endothelial cells, which are the innermost surface lining of blood vessels [27, 28]. Signature

molecules on the endothelial cells that are specific to the volume irradiated can then be targeted. Therefore, a better understanding of the endothelial cells is needed. Knowledge on endothelial cells in normal vessels and in AVM vessels, knowledge on their response to irradiation and particularly the response of AVM endothelial cells to irradiation is to be acquired. This is because endothelial cells differ according to organ, blood flow, location and respond differently to stresses [34, 35]. It cannot be assumed that all endothelial cells respond to radiation in the same way. An understanding of endothelial cells in AVMs and how they respond to radiation will aid in finding a signature molecule that can be targeted for the treatment of AVMs.

(The animal models of AVM have been discussed in detail in a separate section.)

Endothelial cells

The endothelium is a thin monolayer of cells that lines the entire vascular and lymphatic system (Fig. 1.3) [36-38]. The endothelium varies from organ to organ. After the early years of research by William Harvey in circulating blood, attention drew to a network of vessels that physically separated blood and tissue [34, 35]. Later observations by von Recklinghausen found that vessels were lined by cells [35]. Future research involving electron microscopic and physiological studies showed the endothelium as being more than just a barrier [34]. Endothelial cells carry out numerous functions that are critical in nature [39].

The endothelium in a human accounts for $1 - 6 \times 10^{13}$ cells, which is equivalent to almost a kilogram [38, 40]. During early vascular development, endothelial cells organise into vessels in the absence of any vascular system in a process called vasculogenesis. During organ growth, wound healing and metastasis, endothelial cells proliferate from pre-existing vessels in the process called angiogenesis [41]. The vascular endothelial cells emerge from hemangioblasts, which are also the precursor cells for haematopoietic cells [38, 42]. The exact mechanism of this process still remains unclear. Endothelial cells differ in their expression levels and in their response to stimuli in different parts of the vasculature [36, 38, 43].

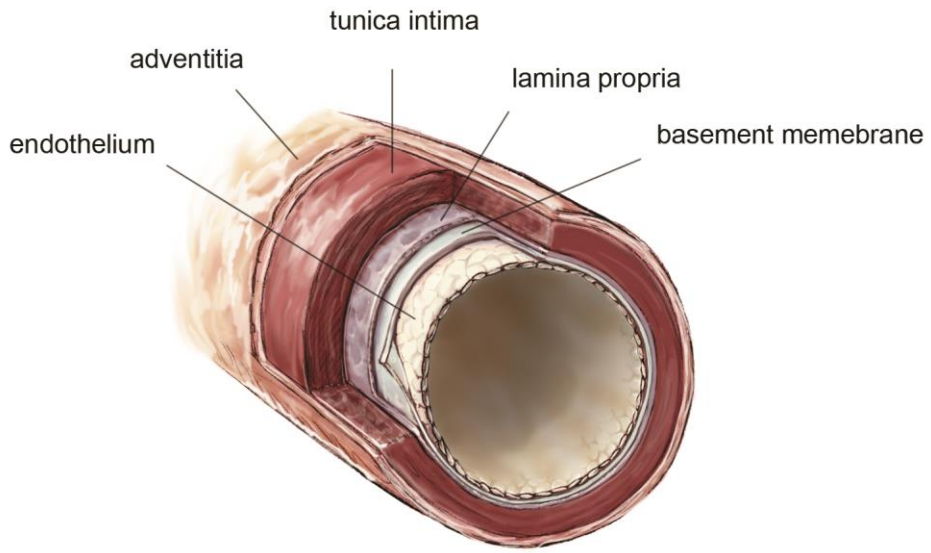

Figure 1.3: Endothelial cells
The thin innermost monolayer of cells in the vasculature is the endothelium (Figure - courtesy of Prof. Stoodley)

Endothelial cells have important roles in coagulation, inflammation and blood vessel structure. In these roles, they produce a wide range of molecules including matrix products such as fibronectin, laminin, collagen and proteases, antithrombotic factors such as thrombomodulin and heparin sulphate, procoagulant factors such as von Willebrand factor, thromboplastin and platelet activating factor, inflammatory mediators such as cell adhesion molecules, factors causing lipid metabolism such as lipoprotein lipase, relaxing and constricting vasomotor factors and growth factors such as platelet derived growth factor, epidermal growth factor and fibroblast growth factor [38].

The electronic microscopic studies from the 1950s showed plasmalemmal vesicles called caveolae and Weibel-Palade bodies [44]. They also showed that the endothelium could either be continuous, connected by a basement membrane, or could have transparent areas called fenestrae, or could be altogether discontinuous where the basement membrane is not connected [35, 45]. This led to the current understanding that endothelial cells vary from one another in structure and function [45]. They express heterogeneity in size, shape, thickness and nuclear orientation from a structural point of view. Endothelial

cells show heterogeneity in the expression of cell surface glycoproteins, proteins and mRNA [46]. They also display heterogeneity in function some of which include transport of nutrients, leukocyte trafficking, permeability, haemostasis, vasomotor tone, cell and nutrient trafficking, angiogenesis, barrier function, osmoregulation and inflammatory responses [35, 36, 38, 43, 46].

Endothelial cell heterogeneity has been studied in detail using various methods, the first of which was scanning electron microscopy (SEM)[44, 47, 48]. Other methods included immunohistochemistry, in situ hybridisation studies and radiolabelled antibodies [49-65]. In the field of proteomics, there is increasing interest focussing on membrane proteins, anti-caveolin antibodies and phage display [66-71]. In the field of genomics, transcriptional arrays and serial analysis of gene expression (SAGE) have been applied [72-76].

AVM Endothelium

The AVM endothelium is different to the endothelium of the normal vasculature [27, 77]. Several studies have been conducted using primary cell culture, surgically resected AVM specimens and animal models.

Culturing of endothelial cells from cerebral AVMs has been a challenging task. Necrosis, post-embolisation, infection and unfavourable *in vitro* conditions are some of the causal factors for unsuccessful AVM endothelial cell culture [77]. Endothelial cell culture from ruptured AVM specimens showed increased expression of endothelin-1 when compared to non-ruptured AVM specimens [78]. The oxyhaemoglobin that is released post rupture interacts with the endothelium thereby increasing endothelin–1 expression [79, 80]. A 25.2 fold increase of the transcriptional repressor, Id1 was reported from gene microarray and quantitative polymerase chain reaction analyses, which has an effect on the downregulation of thrombospondin in AVM endothelial cells [81]. AVM endothelial cells exhibit high proliferation rates with increased rates of angiogenesis [81, 82]. To standardise the function of AVM endothelial cells, thrombospondin was added to AVM endothelial cell cultures to balance the angiogenic phenotype suggesting the molecule may be a potential aid in future

targeted therapies [81]. Interleukin-6 has been reported to produce matrix metalloproteinase 3 and 9 in a similar study [82].

In an immunohistochemical study that involved AVM patient tissues, signal transducers and activators of transcription (STATs) 1 and 3 showed increased expression levels in the endothelium of all the samples [83]. Nestin that has been reported to show increased expression in tumours showed increased expression levels in the endothelial cells of 13 out of 20 AVM tissues [84].

Animal model studies in mice report increased activity of the notch-signalling pathway [85]. This is accomplished by notch ligands Jagged 1 and 2 in the arterial and venous endothelium of the AVM vasculature [86]. In another study, the expression levels of Notch 1 and 4, Delta-like 1 and 4, Jagged 1 and HES1 were examined in a rat arteriovenous fistula model before and after radiosurgery. The drop in expression levels was monitored by immunofluorescent analysis of the endothelium over a period of 42 days [87].

Endothelial Dysfunction

The endothelium exercises various functions ranging from vasomotor tone to mediating inflammation and cell growth [38, 88]. A diverse collection of substances is secreted from the endothelium for the proper maintenance and functioning of the endothelial cell [38]. The repertoire of some of the substances secreted has been discussed in the previous section. When the level of the substances expressed gets disturbed in a measure that is able to alter the phenotype of the cell, the normal, healthy endothelium becomes a damaged endothelium [89-92]. The process is called endothelial dysfunction. It is prompted by a number of physiological factors such as shear stress, ageing, hypoxia and tumultuous blood flow [93, 94]. The most important among those listed above is shear stress because it is that factor that stimulates the endothelium to secrete vasoconstricting factors, prothrombotic agents, procoagulants and adhesion molecules [91, 92].

The equipoise of the endothelium can be interrupted due to an extensive list of reasons [95]. This could be a decrease in the production of vasodilatory

mediators such as nitric oxide (NO) and prostacyclin or an increase of vasoconstrictors such as angiotensin or endothelin [96].

Among the many vasodilators, NO produced from L-arginine plays a crucial role in vasomotor tone [97-99]. NO is produced by nitric oxide synthase (NOS) which has three isoforms – neuronal NOS, endothelial NOS and inducible NOS [96, 100, 101]. The NO from endothelial NOS spreads either through the cell membrane to go about its usual function of paracrine signalling or it can interact with superoxide dismutase which ultimately ends up in the inactivation of NO [96, 102]. Reduction of NO therefore is a common lead up to endothelial dysfunction [96]. Prostacyclins involved in vascular smooth muscle relaxation can employ vasoconstricting properties when expressed in reduced levels due to cytokines, thus resulting in endothelial dysfunction [103, 104]. Angiotensin aids improved endothelial permeability through its production of vascular endothelial growth factor receptors and matrix metalloproteinases. Elevated levels of angiotensin exhibit vasoconstricting properties that lead to endothelial dysfunction [105-108]. Endothelin, under normal conditions increases production of endothelial NOS which synthesises NO. Increased levels foster secretion of adhesion molecules that leads to endothelial dysfunction [109-111].

Inflammation plays a critical role in the pathophysiology of various cardiovascular diseases [112]. Inflammation is characterised into acute and chronic inflammation. This section of the literature review however looks at inflammation as a general process and does not distinguish between acute and chronic inflammation. Rigid adhesion of circulating leukocytes to the endothelium is reduced when the latter becomes inflamed. The resulting condition is referred to as endothelial dysfunction [93, 112, 113]. Some of the causative agents of inflammation include an atherogenic diet, obesity, hypercholesterolemia and hyperglycemia. These agents stimulate the expression of several adhesion molecules that bind to monocytes and T-lymphocytes in the blood stream, resulting in the recruitment of leukocytes and their endothelial transmigration [93, 112, 113]. Endothelial dysfunction occurs mostly at the initiating stages of the inflammatory diseases [114].

Endothelial dysfunction has been evaluated by measuring adhesion molecules such as ICAM-1, VCAM-1, E-selectin and P-selectin, endothelial function markers such as endothelin, von Willebrand factor, tissue type plasminogen activator and plasminogen activator -1, plasma and urine tests for nitric oxide and positron emission tomography [89, 92, 115].

Endothelial Microparticle (EMP)

The lipid bilayered membranes of endothelial cells must be intact for the proper functioning of the cell [116, 117]. When the cell membrane undergoes structural changes due to activation or injury, it results in its dysfunction [116]. Structural changes may involve alterations in the cytoskeletal arrangements and membrane blebbing (also known as membrane budding) [118, 119]. The changes in the bilayer are carried out by the enzymes, flippase, floppase and scramblase [117]. Membrane blebbing may result in release of part of the cell membrane. Such isolated fragments of the membrane are known as microparticles [116].

Endothelial microparticles are vesicles that typically range between 500 nm and 1 μm in diameter [118-123]. The precise mechanism of microparticle release is not very clear [124]. What is known of the process is that during the process of membrane blebbing, part of the cell membrane pinches off as free flowing microparticles into the plasma or extracellular fluid [117, 124]. EMPs are generated as a result of either activation or apoptosis [117, 124]. EMPs are heterogeneous due to the proteins present on their surface. From the parent cell, microparticles carry along with them endothelial proteins such as cadherins, ICAM, PECAM, endoglin, E-selectin and integrin [117, 125].

There is a growing interest of late in the pathophysiological role of EMPs. Under normal conditions, microparticles circulate at concentrations of more than 10^9/mL [126]. The concentration is increased under various pathological conditions [127]. It is not known if EMPs are the cause for the pathological state or if they are released as a consequence of the disease [128]. The presence of EMPs however points to endothelial damage [129].

Following the findings of elevated EMPs in patients with lupus anti coagulant by Combes *et al*, several studies were carried out on various other pathological conditions including systemic, haematological, cardiovascular, atherosclerotic and arterial diseases [116, 130]. EMPs were found to be elevated in acute coronary syndrome, metabolic syndrome, diabetes, hypertension, sickle cell disease, thrombotic thrombocytopenic purpura, paroxysmal nocturnal hemoglobinuria, multiple sclerosis, pulmonary arterial hypertension and kidney failure [131-139]. In thrombotic thrombocytopenic purpura for instance, the level of the EMPs was found to be elevated much before the onset of the condition, allowing EMPs to be used as a potential marker for the condition [135].

Detection of EMPs

The major assays carried out for the detection of EMPs include flow cytometry, immunoassays and nanoparticle analysis [124]. Each assay has its own advantages and limitations.

Flow cytometry is considered the most accepted assay for the detection and quantitation of microparticles. The detection of microparticles using flow cytometry has been widely discussed in literature critically discussing different protocols and pitfalls associated with the technique. Proteins (antibodies) of interest are conjugated with fluorophores for successful detection and quantitation of microparticles based on their size. Flow cytometry is the only technique that can quantify microparticles. Because microparticle research is still in its cradle stage, there needs to be done a lot of standardisation in the methods that involve isolation and fluorescent labelling. As part of standardising the methodology, research has been carried out on key variables such as needle gauge, anti-coagulants used during sample collection, centrifugation speed and temperature for sample storage [140-143].

Flow cytometry cannot however be precise in detecting microparticles that are 100 – 400 nm in diameter as this size range could easily pick up background noise and could also be challenging for antibody binding [124].

ELISA on the other hand is an efficient immunoassay in detecting antigens on the surface of microparticles for successful antibody binding. This is also a cost effective technique when compared to flow cytometry. But it cannot detect microparticles based on their size. There has been a lot of focus of late on nanoparticle analysis due to its potential to capture microparticles that are 25 – 1500 nm, which cannot be detected by flow cytometry [124].

Vascular Targeting

One of the major developments in the field of biomedical science is to develop techniques for successful imaging and treatment of diseased conditions [144-149]. In the treatment of cancer, pharmaceutical and chemotherapeutic agents tend to gather in the non-pathological tissues and organs with a very decreased level of it reaching the tumour tissues and organs [144]. To overcome this limitation of poor selectivity, drugs have been targeted at specific tumour tissues [67, 150, 151]. A list of biomarkers that distinguish normal and pathological states has been studied for the treatment of cancer from proteomic and genomic analyses [146, 147].

Work on the development of vascular targeting agents (VTAs) for cancer treatment began after tumour vessels were obliterated in mice. This initial study by Juliana Denekamp in 1980s was then confirmed when tumour vessels were successfully blocked when targeted with an antibody specific agent such as anti-tubulin [152]. There is a list of prospective antigen markers or VTAs utilised in cancer therapy. Vascular endothelial growth factor (VEGF) located in angiogenic blood vessels, endoglin, p30.5 and endosialin in proliferating endothelial cells, H–5–2, Lewisy–6 in aberrant endothelial cells and integrins, VCAM–1, E–selectin, CD62E, phosphatidylserine, CD44, hyaluronan in activated endothelial cells are examples of some VTAs [153-163].

By means of their anti–angiogenic property, VTAs however only prevent further development of the tumour by blocking formation of new blood vessels. The supply of blood and oxygen feeding into the tumour vasculature remains undisrupted. This is achieved by vascular disrupting agents (VDAs) such as

flavonoids, colchicine, auristatin, soblidotin and combretastatin which are currently in different phases of clinical trials [152].

During the process, an effector molecule is conjugated to an antibody and targeted at a specific site in the vasculature of the tumour cells which will lead to cell necrosis and death [164, 165]. Membrane proteins expressed on the luminal surface of endothelial cells are constantly exposed to those agents circulating in the plasma which make them desirable loci for effector molecules to be targeted [164, 166, 167]. This ligand-based strategy has increased interest in the endothelium as a target site for therapeutic delivery [164, 168]. Histocompatibility complex attached to ricin was the first successful tumour vasculature targeting. Burrows and Thorpe reported this in animal models in 1993 although Folkman had projected the theory much earlier in 1971 [153, 165, 168, 169].

Molecules are targeted at the endothelium through the blood stream using biocompatible drug carriers. This is to overcome challenges of them becoming inactive and also to optimise the effective delivery and binding of molecules [166]. One of the possible options is the successful delivery of molecules contained in nanoparticles to the endothelium [170]. Affinity moieties are selected on the endothelial surface so that molecules along with their carriers successfully bind to the endothelium [166]. A series of potential targets have been studied for this cause in various diseased states such as inflammation, ischaemia, edema, thrombosis, diabetes, oxidative stress, atherosclerosis and hypertension to name a few where the endothelium is targeted for drug delivery [166]. Transmembrane glycoproteins such as angiotensin-converting enzyme, thrombomodulin, PECAM and ICAM are continually expressed in endothelial cells and are escalated in pathological conditions [166]. Of more interest are the molecules such as E–selectin, P–selectin and VCAM that are completely non–existent in normal vasculature and present in pathological vasculature [166].

In the field of oncology, vascular targeting is used in conjunction with other therapies such as radiation therapy, chemotherapy and hyperthermia [153, 171-173]. Advancements in the fields of proteomics and transcriptomics assist in the validation of various targets and call for more work to be done in taking this forward to clinical trials.

The technique of vascular targeting is attractive for treating AVMs that cannot be obliterated with existing treatment modalities. In AVMs, vascular targeting aims to create vascular occlusion by targeted thrombosis in contrast to cell death and necrosis in cancer (Fig. 1.4) [164]. Thrombosis could either be ligand-based or non-ligand based [174, 175].

Research confirms there are differences between the AVM and normal endothelia [176, 177]. Differences are not sufficient though to be exploited for drug delivery [178]. Stereotactic radiosurgery has been used therefore as a priming technique to differentiate between the AVM and normal endothelia [27, 28, 179]. Its precision in targeting AVM vessels without having an effect on the surrounding tissues makes it the preferred modality to be used in conjunction with vascular targeting.

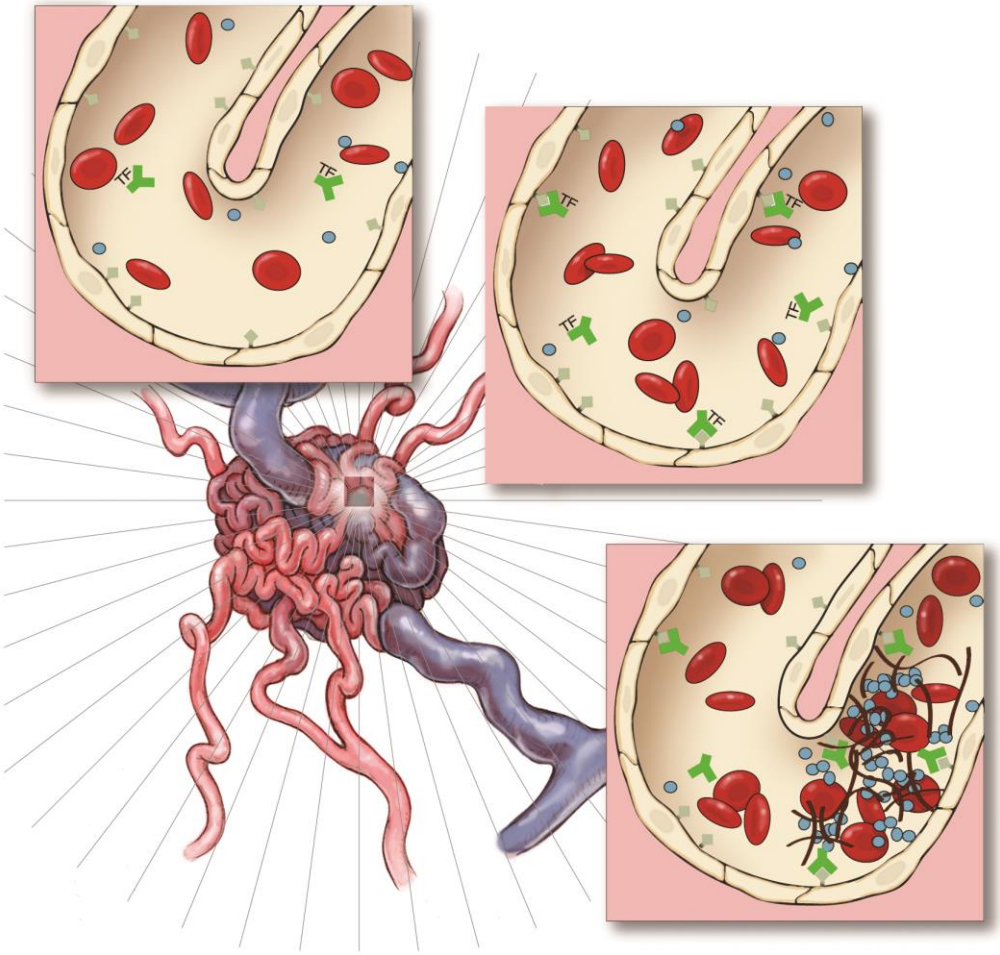

Figure 1.4: Vascular targeting
Radiation induces changes in endothelium cells causing the expression of specific proteins on the surface of endothelial cells. A ligand such as tissue factor (TF) that is attached to an effector molecule binds to the protein of interest and creates intravascular thrombosis. (Figure - courtesy of Prof. Stoodley)

Ionizing radiation causes damage of the DNA and endothelium, which may lead to cell death resulting in the externalisation of certain proteins [180]. In this way, the molecular changes in the endothelial cell surface of AVM vessels could be targeted with pro–thrombotic agents. Consequently, the pro–thrombotic molecules will bind to specific sites in the AVM vasculature leading to rapid thrombosis and AVM occlusion [180].

Storer *et al* reported the first study on vascular targeting in combination with radiosurgery. This was a non-ligand based strategy for enhancing thrombosis in a rat model of AVM using lipopolysaccharide and soluble tissue factor [62]. A ligand-based strategy is more advantageous because it gives a wider repertoire

of effector moieties to remodel the endothelium and enhance thrombosis [175]. E-selectin, VCAM, ICAM, PECAM, ESAM, Hmga2, cadherin 5, cadherin 13 and integrin beta 1 are some of the targets explored for ligand based vascular targeting in an animal model of AVM [62, 63]. The externalisation of phosphotidylserine post radiation was studied in cell models of AVM using live cell imaging. The rate of externalisation was explored at different dosages [180]. Targeting the AVM vasculature is currently in its preliminary stages of investigation. Research is underway in the search for the right candidate molecules that will treat AVMs.

Summary

AVMs are congenital lesions that cause brain haemorrhage in children and young adults that result in death or severe neurological deficits. Current treatment options cannot treat large AVMs and those not superficially located, which leads to a need for a new treatment. Knowledge on the molecular biology of AVMs can lead to a novel approach of treating AVMs by the technique called vascular targeting. The endothelium is the preferred target for delivering therapeutic biomolecules because of its accessibility. Through advanced procedures in proteomics, access is acquired to a range of potential targets that are to be validated for possible therapeutic use in future for the treatment of AVMs.

Hypothesis

1. Endothelial molecular changes induced by radiation can be detected in circulating endothelial microparticles.
2. Radiation induces molecular changes in *in vitro* and *in vivo* models of arteriovenous malformation.

Aims

The overall goal of this Ph.D. project was to discover and validate potential target molecules from animal model, *in vivo* and *in vitro* studies.

1. To determine the protein expression of 3 target molecules – integrin V alpha (CD51), TGF–β1 binding protein (CD109) and bone morphogenetic protein 3 (BMP3) in an animal model of AVM after irradiation (Chapter 2).
2. To quantify the level of endothelial microparticles in plasma samples of AVM patients and compare against control volunteers (Chapter 3).
3. To determine the protein expression of human cerebral microvascular endothelial cells in response to radiation and validate 4 target molecules – talin, myoferlin, HADHA and elongation factor 2 (Chapter 4).

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The following (pg 47 – 64) is a reproduction of the published manuscript that reviews animal models of AVMs. It forms a part of the content described in this chapter.

Experimental Animal Models of Arteriovenous Malformation: A Review

Amal Raj J, Stoodley M. Experimental animal models of arteriovenous malformations: A Review. *Veterinary Sciences* **2015**, 2(2), 97-110

Abstract
Arteriovenous malformations (AVMs) are congenital lesions that cause brain haemorrhage in children and young adults. Current treatment modalities include surgery, radiosurgery and embolisation. These treatments are generally effective only for small AVMs. Over one third of AVMs cannot be treated safely and effectively with existing options. Several animal models have been developed with the aims of understanding AVM pathophysiology and improving treatment. No animal model perfectly mimics a human AVM. Each model has limitations and advantages. Models contribute to the understanding of AVMs and hopefully to the development of improved therapies. This paper reviews animal models of AVMs and their advantages and disadvantages.

Introduction

Cerebral arteriovenous malformations (AVMs) are abnormal connections between arteries and veins that lead to the formation of a tangled collection of vessels referred to as a 'nidus' [1-3]. The high-pressure shunt of arterial blood flowing through the fragile vessels in the nidus and the draining veins can cause intracranial haemorrhage resulting in death or disability [4, 5]. The prevalence of this condition is 0.01% – 0.5% of the population with presentation common in children and young adults [2, 6]. In addition to haemorrhage, AVMs can present with epileptic seizures, chronic headaches, migraines and ischaemic neurologic deficits [7-10].

Current treatment options include surgical resection, endovascular occlusion and radiosurgery, or combinations of these [11]. Surgery is generally limited to small AVMs that are superficially located [12-14]. Large AVMs (more than 3 cm diameter) and those located in critical regions such as the thalamus and basal ganglia remain a challenge for effective treatment. There is a higher risk of morbidity following surgery in such cases [11, 15, 16]. Radiosurgery, although attractive as a relatively non-invasive treatment, is often not as effective as surgery [17, 18]. There is also a 2 – 3 year delay to AVM occlusion after radiosurgery treatment and a success rate of approximately only 80% even for small lesions [14, 19-21].

There is an urgent need for a new treatment for the over one third of AVMs that cannot be safely and effectively treated with current protocols [21, 22]. Animal models are required to investigate the pathogenesis of AVM formation and their biological and haemodynamic characteristics. Development of a new effective treatment for AVMs is also likely to depend on the use of appropriate animal models. Improving responses of AVM tissue to radiation, and developing adjuvants such as radiosensitisers, could be best achieved using animal models. Potential new biological therapies such as gene therapy or vascular targeting to induce AVM thrombosis will require animal models for developing and trialling the therapeutic agents. Refining endovascular techniques is a further field where animal models are extremely useful.

Many animal models have been developed in the last three decades. Each has advantages and disadvantages and there is no model that is suitable for all purposes. This review discusses each of the research settings where models are used and discusses the advantages and disadvantages of each model. Standardising animal model use for each research indication would hopefully lead to uniformity in research methods and more efficient progress towards the goal of developing better treatments for AVM patients.

Study of AVM Haemodynamics

Replicating AVM haemodynamics is necessary for the study of AVM pathophysiology and refinement of endovascular techniques. Most models include an arteriovenous shunt and a nidus.

The rete mirabile in swine has been used as an AVM model for its network of microarteries that are interconnected and resemble an AVM nidus, at least angiographically [23]. However its arterial-to-arterial structure does not mimic that of a human AVM, which is arterial to venous [23]. This limitation was addressed by creating a short-term arteriovenous shunt between the rete and the internal carotid artery and the cavernous sinus [23]. Although this model has morphological resemblances to human AVMs, there are several adverse effects on the animals such as proptosis, chemosis and sub-conjunctival haemorrhage. The model lasts for a maximum period of only a week by which time the connections have occluded spontaneously. As with most AVM models, the rete

mirabile model is not inside the brain. The model however is particularly useful for refining endovascular techniques and trialling new embolic agents [23].

In an attempt to simplify the creation of the model, Massoud *et al.*, created an AVM model in the neck of swine, thus avoiding the adverse effects of the rete-cavernous fistula model. In order to increase blood flow in the fistula, three arteries are occluded in the neck region. This is followed by a side-to-side anastomosis forming a carotid-jugular fistula [24].

This model has been criticised for its use of microcatheter and detachable balloons, which are very expensive [25]. Qian *et al.* created a similar model in sheep, but did not occlude the arterial branches (Fig 1.5). The sheep rete mirabile is quite different to that in swine. The retia appear as two units, connected by bridging vessels in sheep and as one in swine. This proves to be an advantage for sheep over swine where it gives the possibility for researchers to study multiple AVMs [25].

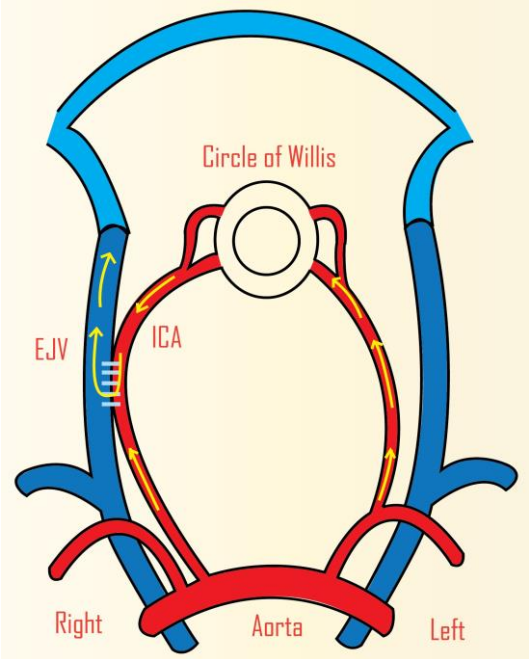

Figure 1.5: Animal model of cerebral AVM in sheep
Side-to-side anastomosis was carried out between the CCA and EJV (Right). The arrows show the direction of the circulatory flow after anastomosis. ICA: common carotid artery, EJV: external jugular vein. (Adapted from Qian et al., 1990) [25].

Swine are preferred for their large neck vessels, which allow for faster and easier fistula creation. There are models in which external arteries need not be occluded [26, 27]. An end-to-end anastomosis instead of a side-to-side anastomosis shows an increase in blood flow in the rete mirabile. Spontaneous occlusion does not occur as in previous models, which means these could be used for research that involves a longer duration [23, 24, 26, 27]. Additional advantages with swine model are that the swine coagulation system is similar to humans and they are inexpensive [24].

Haemodynamic models are also used to study perfusion pressure breakthrough, the role of venous hypertension and the role of thrombosis in AVMs. Rat models are often used for research in these areas [28, 29]. Various haemodynamic arrangements have been used, although the model usually is located in the neck region by creating an anastomosis between the common carotid artery and external jugular vein [6, 30]. In these models, the common carotid artery acts as the feeding artery and the external jugular vein acts as the arterialised vein. In one model, the anastomosis is between the rostral carotid artery and the caudal jugular vein. This arrangement results in intracranial hypoperfusion, analogous to the 'steal' phenomenon around AVMs, and has been used to study the effects of chronic ischaemia [31, 32]. The carotid jugular fistula model by Morgan *et al.* was used to study hypoperfusion. An end-to-end anastomosis was performed between the common carotid artery and the external jugular vein (Fig 1.6). The involvement of both the intracranial arterial and venous systems with the extracranial venous systems is a feature of this model [32].

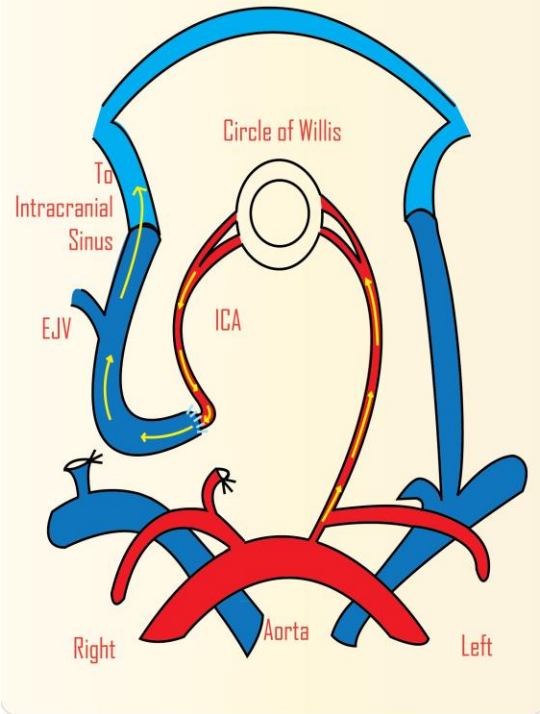

Figure 1.6: Schematic representation of animal model of cerebral AVM in rat
Carotid jugular fistula model that involves an end-to-end anastomosis between the common carotid artery and external jugular vein. ICA: internal carotid artery; EJV: external jugular vein. (Adapted from Morgan *et al.*, 1989) [32].

In other models, the anastomosis is a side-to-end connection between the common carotid artery and the rostral external jugular vein. In this arrangement arterial blood flows through the external jugular vein and its branches (the "nidus") and the outflow is then through the transverse sinus and the contralateral jugular vein (draining vein) (Figure 1.7) [33, 34]. This model has been used to study the molecular and morphological changes in AVMs treated with radiosurgery [6, 35]. Critically, the model produces an endothelial phenotype that resembles that of human AVMs; this is crucial when studying radiation effects because the endothelial response to radiation is different for different phenotypes. Blood flow has been shown to increase with time in this model, at least until 42 days post-surgery [6].

In the many AVM models that claim to have a blood flow similar to what is observed in human AVMs, the model is usually extracranial in location and not intracranial [24, 36, 37]. In response to this, an AVM model was developed in

beagle dogs (Figure 1.8) [38]. An end-to-end anastomosis between the superficial temporal artery and the middle cerebral artery and an end to side anastomosis between the superficial temporal artery and the dorsal sagittal sinus were carried out. A muscle graft supplied by the superficial temporal artery was fixed in the ischaemic region of the brain. Ischaemia was found to be aggravated in the arteriovenous fistula region [38]. An advantage of this model is its intracranial location. This model has not been reported in any subsequent investigations.

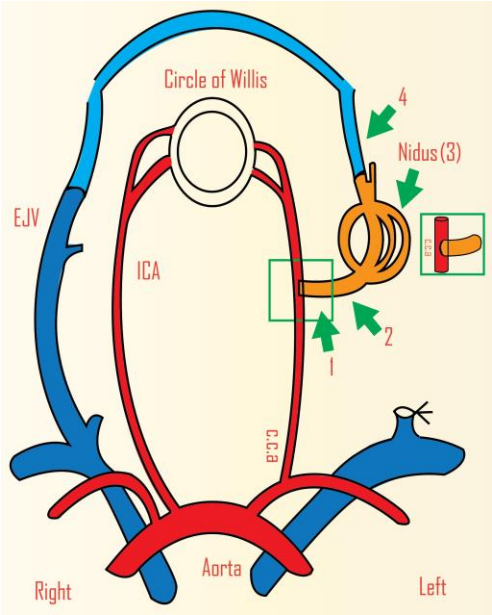

Figure 1.7: Schematic representation of an AVM model in rat
This is a carotid jugular, side-to-end anastomosis between the common carotid artery and external jugular vein. This is a very efficient model for radiosurgery related studies. CCA: common carotid artery, ICA: internal carotid artery, EJV: external jugular vein. Anastomosis (**1**) arterial feeder (**2**) nidus (**3**) draining vein (**4**).

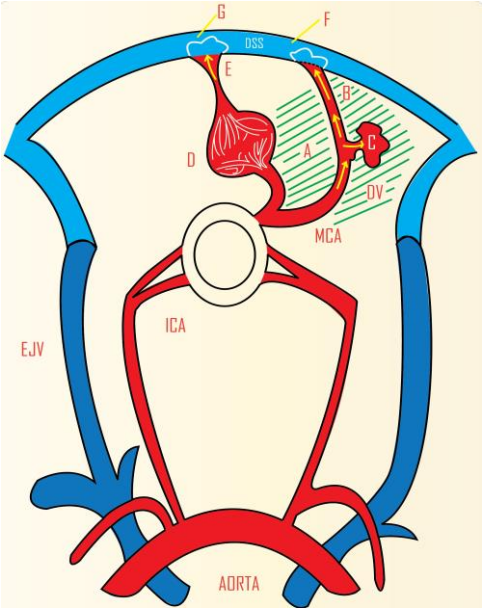

Figure 1.8: Animal model of cerebral AVM in beagle dogs
The dog model located intracranially has the benefit of the vascular capillary network around the AV shunt, resembling the human AVM more closely. DSS: Dorsal sagittal sinus MCA: Middle cerebral artery Area shaded with slanted lines: Relative ischaemia to stimulate angiogenesis. **A**: End-to-end anastomosis **B**: End-to-side anastomosis **C**: Muscle graft implanted in the ischaemic brain tissue and supplied by the bypass **D**: Nomal capillary network in the brain parenchyma **E**: Vein with normal drainage **F:** Arterialised blood **G**: Venous blood. (Adapted from Pietila *et al.*, 2000) [38].

A model was developed by Numazawa *et al.* to study hypoperfusion and impaired CO_2 reactivity. A femoral vein graft was used to connect a cortical branch of the middle cerebral artery and the superior sagittal sinus, thus creating an arteriovenous shunt. This model was shown to closely resemble the haemodynamics of AVM patients with hypoperfusion and decreased CO_2 reactivity [39].

The rete mirabile is a suitable model for the development of endovascular techniques. Surgery is not required for this arterio-arterial system. However for studies involving the pathophysiology of AVMs and their haemodynamic properties, an arterio-venous shunt is required. This swine model can be used for this purpose, however the arteriovenous model in rats has an advantage in terms of simpler animal care and maintenance and is far more economical than swine and sheep.

Radiosurgery

Radiosurgery is one of the treatment options for AVMs [40-42]. A single, focussed high dose of radiation is delivered to a target volume of AVM tissue [42, 43]. Free radicals react with the DNA causing damage [44, 45]. This is followed by endothelial proliferation, smooth muscle cell proliferation and thrombosis, which lead to AVM obliteration [43]. The exact mechanism is still unknown. AVM animal models have therefore been developed to study the short-term effects of radiosurgery.

De Salles *et al.* treated eight swine with 20, 30, 40, 50, 60, 70, 80 and 90 Gy dosages. The rete mirabile was selected as the target tissue. Neurologic dysfunctions, such as abnormal eye movement, were commonly observed in the animals. A decrease in vascularity was observed in animals that received 50 Gy or more dosage and complete obliteration of the rete mirabile was observed in the animal that received 90 Gy [46]. The rete mirabile, which is located in the cavernous sinus, is an arterioarterial system unlike an AVM that is an arteriovenous system [47-49]. This means that that model lacks the high-pressure blood flow in arterialised veins typical for an AVM.

Jahan *et al.* used the model by Massoud *et al.* to study the effects of radiosurgery on a swine model where a carotid-jugular fistula was created in the neck [50, 51]. The preferred dosage was 40 Gy. Decreased vascularity was observed in treated animals when compared to those not treated at a three-month time point [50]. Histopathological changes post-radiation were studied. The study showed expressions of Type IV collagen to be very similar to those in resected specimens post-radiation from humans [50].

Radiosurgery is shown to cause vascular obliteration in several models. Intimal hyperplasia, necrosis, oedema and gliosis have been observed in the tissues surrounding the target volume [46]. In the rete mirabile model for instance, these could be pointed to as the main cause for several neurological deficits such as hemiparesis, loss of coordination, disruption in vision and seizures [46]. Mut *et al.* studied a carotid jugular fistula model in a rat to see the effects of radiosurgery

in the tissues surrounding the target volume for a dosage of 25 Gy [43]. DNA strand breaks and the level of free radicals were analysed. There was significant apoptosis in irradiated groups irrespective of the presence of the fistula [43].

Great care is taken in the complete obliteration of AVMs during surgical excision. A small amount of tissue left behind is capable of AVM angiogenesis. This applies to the radiosurgery treatment modality as well. AVM angiogenesis after radiosurgery is highly dose dependent according to the corneal angiogenesis model study [52]. Resected AVM specimens from humans were implanted in the corneal tissues of rats. Dosages of 1.5 Gy, 3 Gy, 15 Gy and 30 Gy were administered to the animals. The latter two dosage groups showed a greater anti-angiogenesis effect than the former two [52].

Another major application radiosurgery models have is in the search for a potential vascular target for inducing thrombosis. In the field of cancer, vascular targeting has been successful in inducing thrombosis due to the molecular differences that distinguish tumour vessels from normal vessels [33]. This however requires the AVM vessels to differ significantly from normal vessels. Tu *et al.* have reported increased levels of ICAM, VCAM and E-selectin in human AVM endothelium when compared to the endothelium in normal vessels, however the changes were not sufficiently discriminating to be used as a vascular target [53]. The carotid jugular anastomosis rat model was to see if radiosurgery induced molecular changes sufficient to discriminate them from normal vessels [53]. In this model, the external jugular vein was anastomosed to the common carotid artery. After six weeks when the fistulae were considered mature, AVM niduses were treated with radiosurgery (25 Gy). In this work, radiosurgery was given not as a treatment for the AVM but rather as a priming technique to explore the endothelial molecular changes that would come about as a result of radiosurgery [54]. Subsequent studies have used the model to demonstrate induced thrombosis with vascular targeting [33, 35].

Swine and rat models are highly suitable to study the effects and efficacy of radiosurgery. The swine model is resource intensive and suffers from the fact

that the 'nidus' (rete mirabile) is purely arterial. The rat model is more practical and consists of arterialised veins that more closely resemble the molecular characteristics of human AVMs.

Genetic Studies

There is a growing interest in gene therapy to treat large AVMs and those located in regions such as the thalamus, basal ganglia and brainstem that cannot be treated by surgery [55-58]. For this to be successful, a family of molecules or a particular gene responsible for a certain characteristic feature such as narrowing of the arteries needs to be identified [58, 59]. Once identified, that particular family of molecules or the gene may be overexpressed to remove the AVM successfully [58]. AVMs are typically sporadic [60]. However, 2% of AVMs are familial, including patients with hereditary haemorrhagic telangiectasia (HHT) or Rendu-Osler Weber syndrome [60-62]. In HHT, abnormal malformations in different organs such as the lung, liver, brain and spine occur as a result of mutations in the endoglin and ACVRL1 genes [63, 64]. The causal factors for this genetic disorder are unknown [65]. This poses the need for HHT animal models to better understand the pathophysiology of HHT and also to develop new therapeutic modalities. Animal model studies have successfully been able to identify certain genetic mutations and risk factors [2, 66]. The TGF-β genes are a good example of those associated with non-sporadic AVMs. The TGF-β genes go through a loss of function mutation in endoglin and activin like kinase giving rise to the HHT (1 and 2) mutation resulting in AVM formation [65, 67, 68].

In the first transgenic mouse model study by Satomi *et al.*, ten endoglin heterozygous mice ($Eng^{+/-}$) were compared with 15 controls ($Eng^{+/+}$) mice. AVMs are believed to develop in the mice that are heterozygous at the endoglin locus [69]. Only 30% showed abnormalities that closely resemble those present in human AVMs. However, no abnormalities were observed in any of the control animals. Changes such as decreased arteriole constriction and increased relative dilatation were also observed. More research is required to determine if these changes actually contribute to the formation of AVMs in HHT patients [70]. In another mouse model that was stimulated with VEGF, 89% of the $Eng^{+/-}$ mice showed vascular abnormalities and none were observed in the controls [71]. The

lung, liver and intestine showed dilation in some vessels with no external signs in one particular animal [70]. Another animal that had an ear telangiectasis showed an AVM nidus that resembled those in human and canine brain [70]. Despite the fact that $Eng^{+/-}$ and $Acvrl1^{+/-}$ mice are the best available HHT models at present, past studies do not show the HHT features at high frequency [69, 72, 73]. Although the two studies could be criticised as not being true AVM models and for their small sample size, they may contribute to research on the abnormal phenotype of AVMs in humans [66].

A transgenic arteriovenous fistula between the common carotid artery and external jugular vein with TAo from a donor mouse was created in 112 rats to investigate a possible gene therapy for AVMs. Grafting however would impose the challenge of not being able to investigate certain genetic changes in the fistula [58]. Limitations such as low lesion frequency in $Eng^{+/-}$ mice and lack of the human AVM phenotype in currently existing models call for more work to be carried out in this area of study. Two HHT 1 animal models were created to study the developmental and adult onset of cerebral AVMs [74]. *Eng* conditional knockout mouse lines were obtained by crossing $Eng^{2fl/2fl}$ with R26CreER, SM22α-Cre and LysM-Cre. The $Eng^{2fl/2fl}$ were crossbred with SM22αCre to study the effects of embryonic *Eng* deletion on the post-natal development of AVMs [74]. Almost 90% from the group $Eng^{2fl/2fl}$; SM22α-Cre showed the AVM phenotype. No lethality was observed until the fifth week [74]. In previous similar models involving a conditional knockout of the Alk1 gene, intracranial haemorrhage led to lethality by even post-natal day 5 and two weeks limiting the use of such models in studies related to new therapies [75, 76]. The $Eng^{2fl/2fl}$; R26CreER were given a dose of tamoxifen for three consecutive days to induce *Eng* deletion. Those mice that were additionally injected with adeno-associated viral vector expressing VEGF showed AVM phenotype in the form of lesions eight weeks after *Eng* deletion. No lethality was observed in the $Eng^{2fl/2fl}$; R26CreER group upto two months post-tamoxifen treatment [74].

Transgenic animal studies are useful for investigation of gene changes. Any potential gene therapy would need to be trialled on such models. The major drawback with these models is in identifying AVMs in individual animals.

Conclusions

Animal models have been developed because naturally occurring AVMs in animals are quite rare. There is no animal model that is perfect with all the necessary characteristics. The choice of model will depend on the purpose of the research. The rete mirabile is suitable for angiographic studies, but the lack of arterialised veins makes the model unsuitable for studies of AVM biology. Models with an arteriovenous shunt are useful for studying the biology of arterialised veins and for investigating the effects of cerebral hypoperfusion. Investigations using radiosurgery should use a model with arterialised veins and an identifiable model nidus. Transgenic models are appropriate for research related to HHT and for genetic studies.

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Chapter 2

Expression of CD51, CD109 and BMP3 in an animal model of arteriovenous malformation after radiosurgery

The work described in this chapter was presented at the following conference, Amal Raj J, McRobb L, Lee V, Wiedmann M, Grace M, Moutrie V, Stoodley M. Molecular changes in arteriovenous malformations treated with radiosurgery. Digital Poster presented at the 'AANS/CNS Joint Cerebrovascular Section Annual Meeting', Los Angeles, United States of America, February, 2016

Abstract

Background: Cerebral arteriovenous malformations (AVMs) are the most common cause of brain haemorrhage in children and young adults. Over one third of AVMs cannot be treated using current methods. There is a need for new treatments. The overall goal is to use radiation to induce molecular changes in endothelial cells lining the AVM vessels that could be used as candidates for vascular targeting to create selected thrombosis.

Methods: In a rat AVM model, 30 rats had an end-to-side anastomosis between the external jugular vein and the common carotid artery. Half the rats received a 20 Gy dose of radiation by GammaKnife and half were sham-irradiated. AVM tissues were extracted on days 1, 3, 5, 7, 21 and 42 post-irradiation and immunohistochemical analysis on frozen sections was performed to study the localisation and expression of CD51, CD109 and BMP3 after irradiation.

Results: CD51 was present in both irradiated and control AVM tissues. CD109 was not present in either irradiated or control AVM tissues. BMP3 was present in the day 21 irradiated AVM tissues.

Introduction

Brain arteriovenous malformations (AVMs) cause intracranial haemorrhage in children and young adults as a result of high-pressure blood flowing through the fragile cerebral veins [1-4]. Haemorrhage leads to morbidity or mortality [5]. Surgical resection is the most effective way of treating AVMs to prevent haemorrhage, but is associated with high risks when treating large and deep lesions [6-8]. Another mode of treating AVMs is radiosurgery, which gradually occludes the vessels over a period of 2 – 3 years, although there is no guarantee of fully obliterating them [9-12]. Over one-third of AVMs cannot be treated with currently existing treatment options [13-17]. There is therefore an urgent need to find new treatments for such AVMs.

The technique of vascular targeting, which is used in cancer treatments, is attractive as a method of occluding AVM vessels [13, 18, 19]. Vascular targeting requires a target molecule. Such a molecule has not been identified on the AVM endothelium. Therefore radiosurgery has been proposed as a method of priming the AVM endothelium to create a molecular target. [20-22]. In ligand-based vascular targeting, an effector molecule that is bound to a ligand could target such differentially expressed protein molecules on the endothelial cell surface to induce thrombosis, thus obliterating the AVM [13].

It has been established that radiation impacts the expression of certain coagulation and thrombosis related protein molecules [23-26]. In studies on the molecular biology of AVMs, certain molecules have been found to be increased in expression in the AVM endothelium, although none of them are sufficiently discriminating from the normal endothelium [27-31]. The difference in the protein complement of the two endothelia is crucial to successfully target and enhance thrombosis in the AVM vessels. The expressions of integrin V alpha (CD51), TGF-beta-1-binding protein (CD109) and bone morphogenetic protein 3 (BMP3) have been found to be increased in expression in an earlier *in vitro* study on brain endothelial cells post-irradiation [32]. It is not clear if the changes in the expression of the targets identified in the *in vitro* study also apply to *in vivo* samples. Their expression on AVM endothelial cells needs to be explored to

further validate these molecules as potential vascular targets. CD51 was a successful marker in capturing endothelial microparticles using flow cytometry (Chapter 3) and hence was selected for this study. CD109 and BMP3, which play a pivotal role in the TGF-β pathway were detected in previous *in vitro* proteomic studies and hence selected for the following study.

The aim of this study was to investigate the expressions of CD51, CD109 and BMP3 in an animal model of AVM. This model mimics the haemodynamics of the blood flow in a human AVM and will be used to study radiation-induced protein expressions. The significance of the expression levels in the AVM 'nidus' and the surrounding vessels will also be explored.

Methodology

Animal model

All animal experiments were carried out with approval from the Animal Care and Ethics Committee, Macquarie University, Sydney, Australia. An end-to-side anastomosis was performed between the external jugular vein (EJV) and common carotid artery (CCA) as described previously (Fig. 2.1) [33, 34]. Surgeries were conducted in Sprague Dawley male rats that were seven weeks old and weighed 284±32g.

Radiosurgery

Rats that had had surgery were irradiated at six weeks (n=3). The animals were irradiated at the Gamma Knife facility at Genesis Care, Macquarie University Hospital, Macquarie University, Sydney, Australia. This has been described in detail previously [33]. AVMs were localised by axial full body CT scan prior to irradiation. Rats in the control sample underwent similar surgery to those in the treatment group, but were not subjected to radiation (n=3).

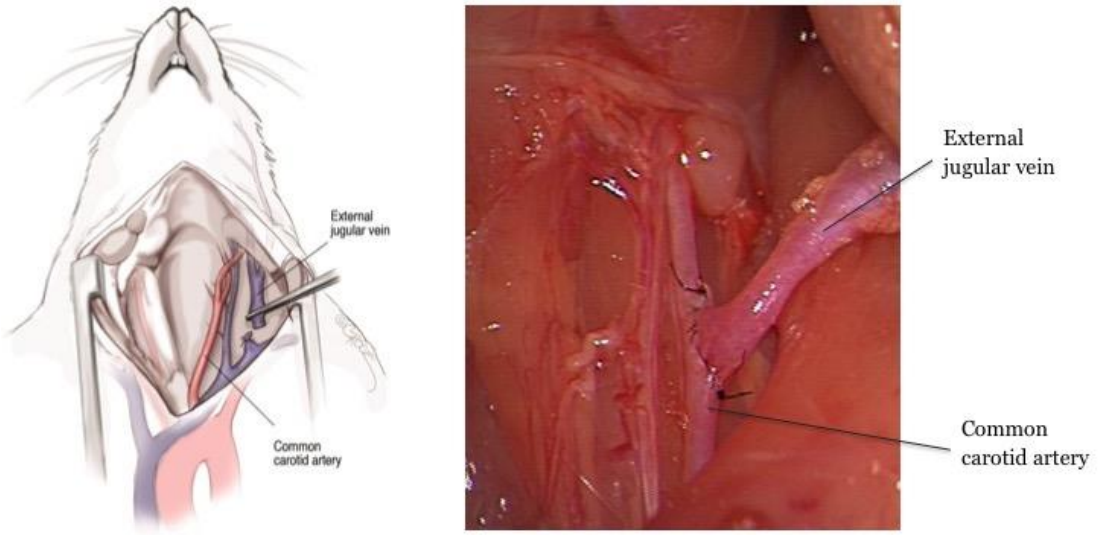

Figure 2.1: Animal model of AVM
An end-to-side anastomosis between the EJV and CCA was performed. (Figure – Courtesy of Prof. Stoodley and Dr. Raoufi-Rad)

Tissue extraction

At days 1, 3, 7, 21 and 42 post-irradiation, animals were perfused with 500 mL of phosphate buffer saline (pH 7.4). Each time point had a total of six rats, where three rats were irradiated and the other three were sham control rats. The AVM

vessels and CCA were extracted and placed in cryomolds with optimal cutting temperature compound. The cryomolds were then snap frozen in liquid nitrogen and stored at -80° C for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical analysis was performed on 10 μm sections of the AVM vessels and CCA. The sections were fixed with 2% paraformaldehyde for 10 min and blocked with foetal bovine serum (Sigma Aldrich, Catalogue #12007C) for 1 hour at room temperature. Protein localisation was investigated for CD51 (BD Biosciences, Catalogue #611013), CD109 (Santa Cruz Biotechnology, Catalogue #sc-98793) and BMP3 (Santa Cruz Biotechnology, Catalogue #sc-9031). The sections were incubated at 4° C overnight with antibodies against the three proteins. To help in the localisation of the endothelium, the sections were co-stained with PECAM (CD31) (BD Biosciences Catalogue #555025). Donkey anti-rabbit AlexaFluor647 secondary antibody (Life Technologies Catalogue #A31573) was used to visualise CD51, CD109 and BMP3 and donkey anti-mouse AlexaFluor488 was used for CD31 ((Life Technologies Catalogue #A1202). The sections were stained with Hoechst (5 μg/mL) for visualising nuclei. Images were obtained from the Olympus FV-1000 confocal microscope and viewed using the FluoView software.

Results

CD51

In the AVM tissues, CD51 showed increased signal intensity in the endothelial cells on day 1 after irradiation. CD51 can be seen throughout the endothelium. The level of fluorescence intensity was decreased on day 3 after irradiation. By day 7 after irradiation, CD51 intensity was more patchy and reduced compared to day 1. Signal intensity at day 21 was very fragmented on the endothelium, similar to day 7. No expression was observed in the control tissues of days 1, 3, 7 and 21. By day 42, the irradiated tissues showed no CD51 signal, similar to that of control tissue samples (Fig. 2.2).

In the CCA, CD51 was observed in both irradiated and control tissues at all time points. Signal intensity was fragmented in the endothelium, similar to the irradiated AVM tissues on days 7 and 21. Increased intensity was observed in irradiated tissues on day 7 where co-localisation was observed right through the endothelium. Low intensity was observed on day 21 and the irradiated samples looked the same as the controls. Irradiated tissues on day 42 showed an increase in fluorescence intensity, similar to the irradiated samples on day 7. However co-localisation can be observed in the control samples in a disjointed pattern in both days 7 and 21 (Fig. 2.3).

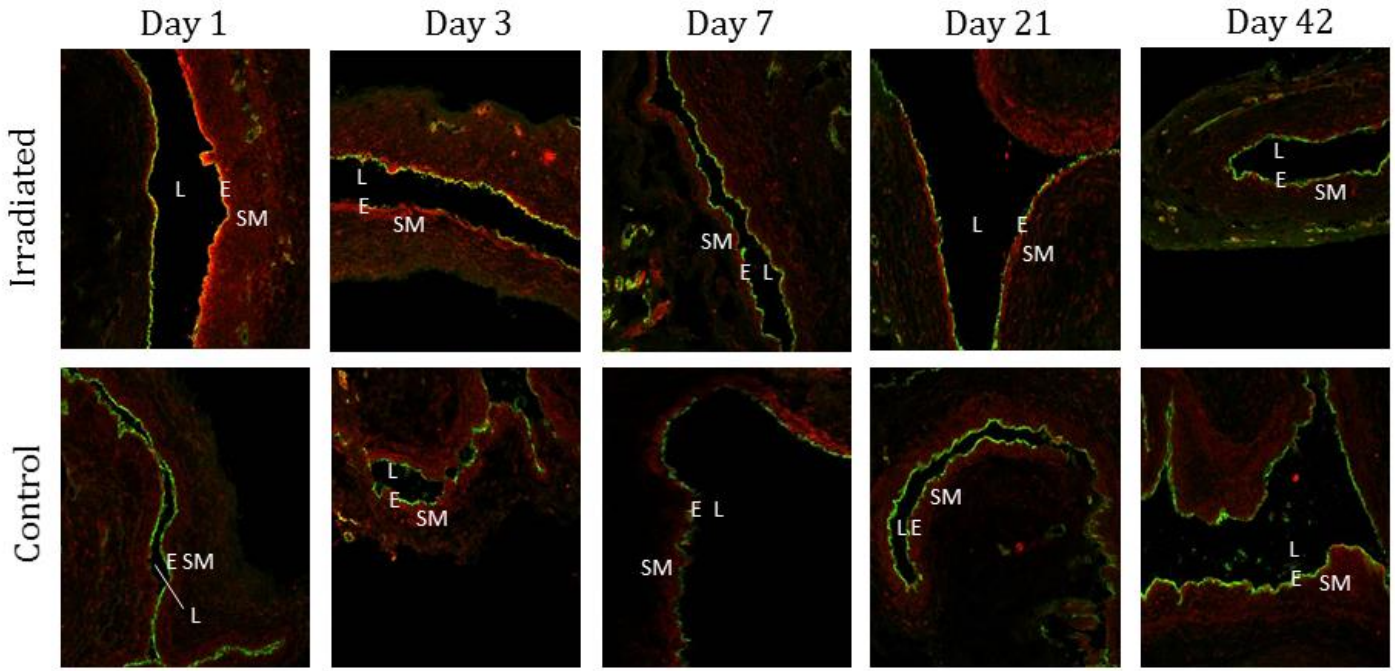

Figure 2.2: CD51 signal intensity in irradiated and control AVM tissues
L – lumen, E – endothelium, SM – smooth muscle cells. CD51 present in both control and irradiated tissues.
CD51 – AlexaFluor647; CD31 – AlexaFluor488

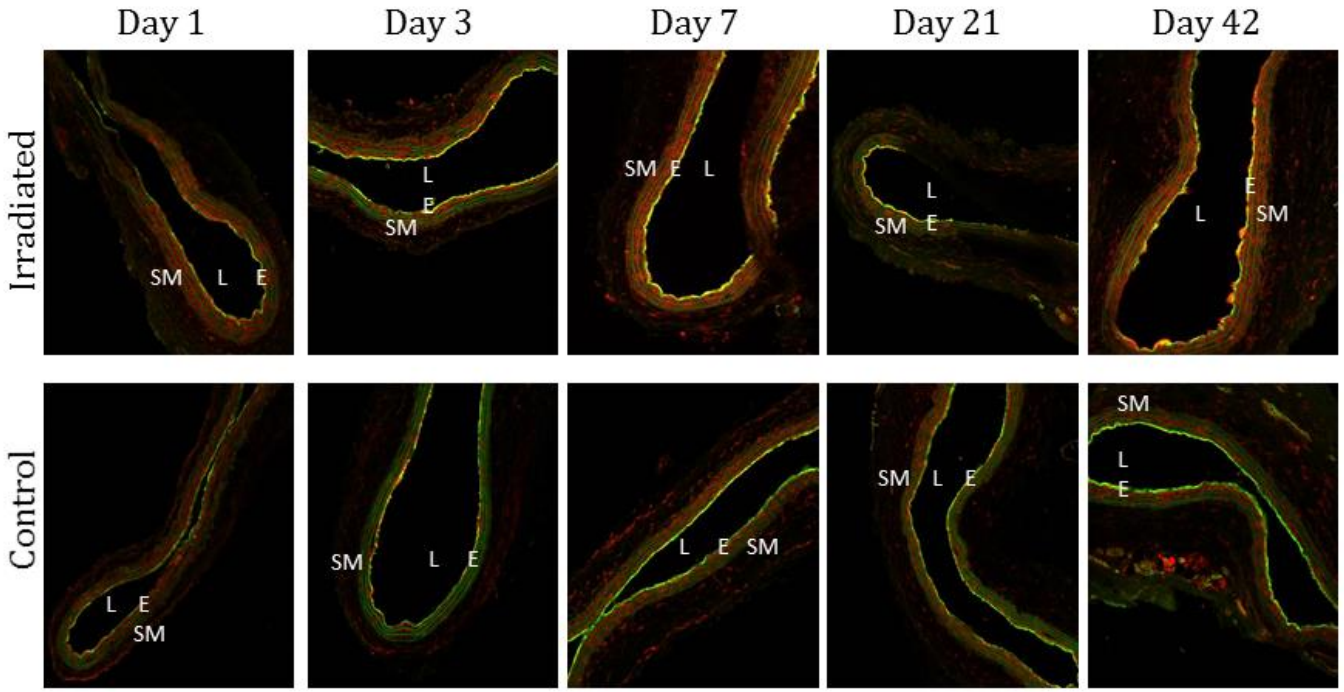

Figure 2.3: CD51 signal intensity in irradiated and control CCA tissues
L – lumen, E – endothelium, SM – smooth muscle cells. CD51 present in both control and irradiated tissues.
CD51 – AlexaFluor647; CD31 – AlexaFluor488

CD109

In the AVM tissues, co-localisation was observed in one of the three rat tissue samples on day 1 after irradiation. The other two samples showed no co-localisation and looked very similar to the control samples. No signal was observed on days 3, 7, 21 and 42 in both irradiated and control groups (Fig. 2.4).

In the CCA, no signal was observed on days 1, 3, 7, 21 and 42. There was no difference between the control and irradiated tissues (Fig. 2.5).

There was no signal observed in the endothelium and in the smooth muscle cells right throughout, other than in the endothelium of the one AVM sample from the day 1 time point.

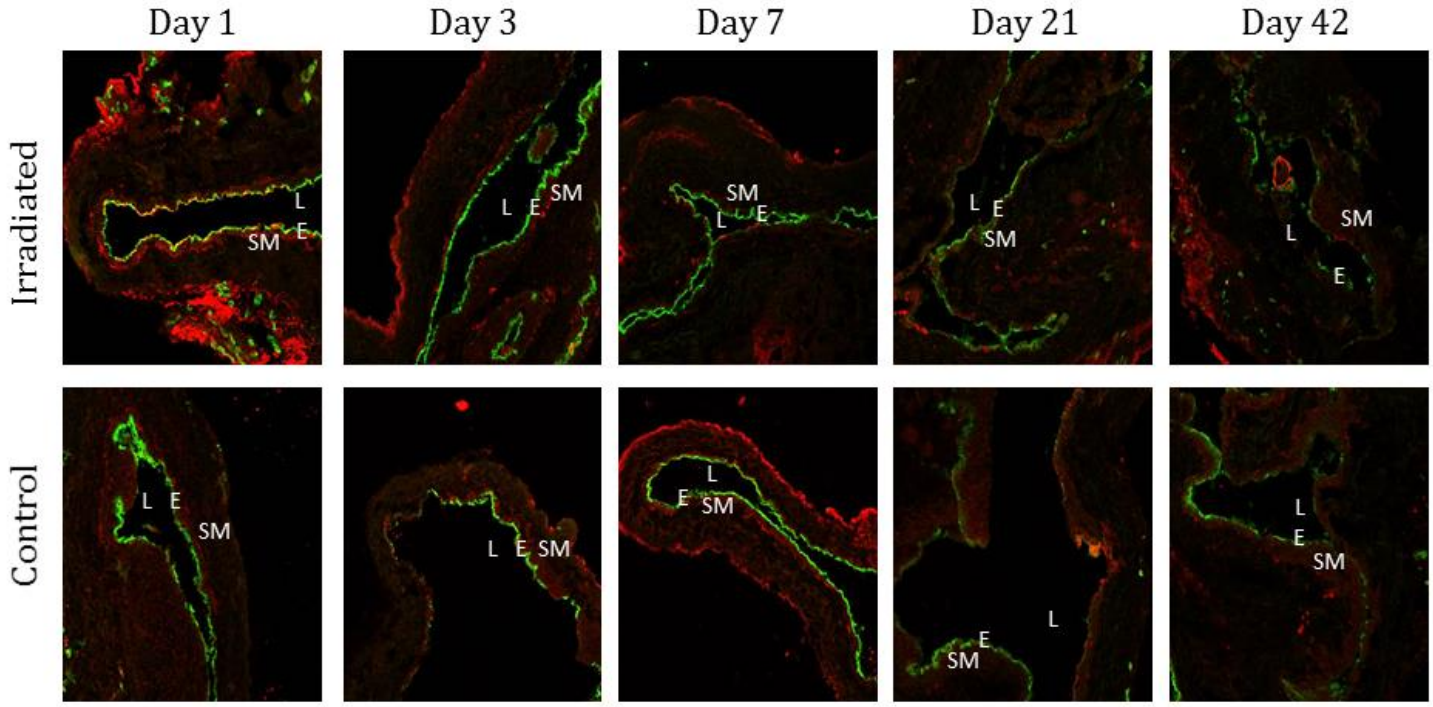

Figure 2.4: CD109 signal intensity in irradiated and control AVM tissues
L – lumen, E – endothelium, SM – smooth muscle cells. CD109 absent in both control and irradiated tissues.
CD109 – AlexaFluor647; CD31 – AlexaFluor488

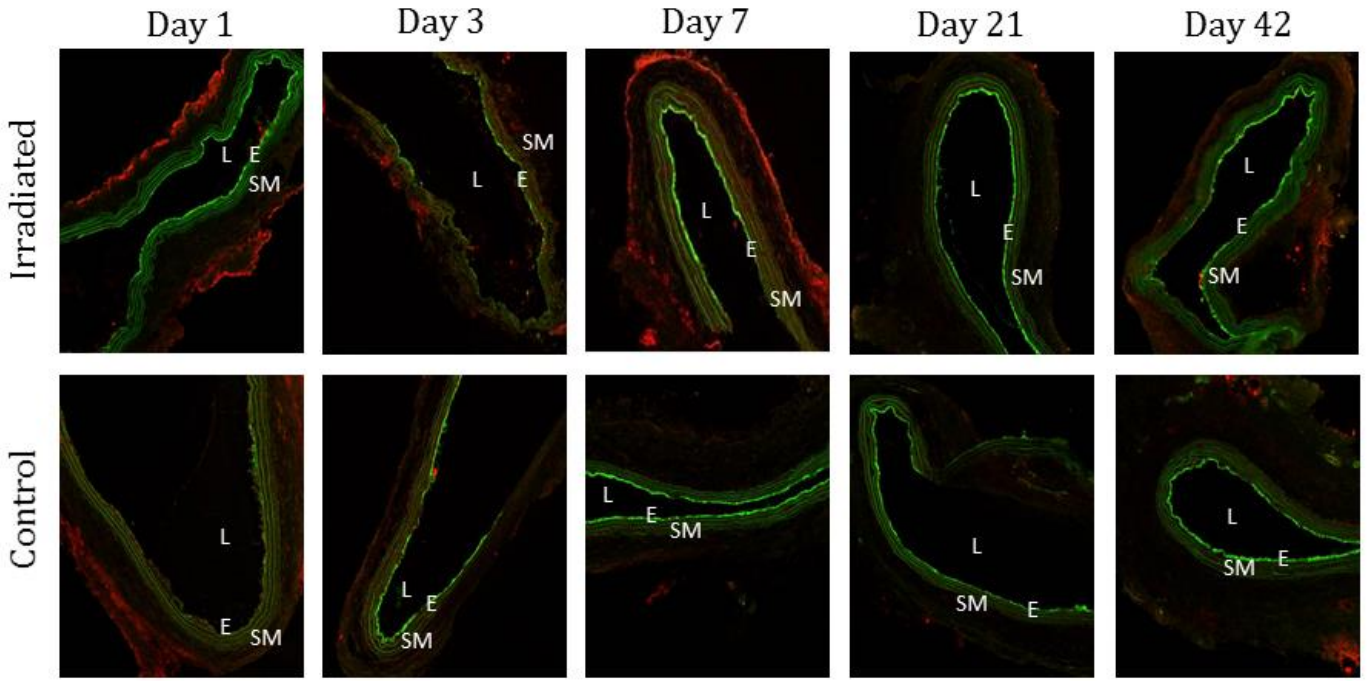

Figure 2.5: CD109 signal intensity in irradiated and control CCA tissues
L – lumen, E – endothelium, SM – smooth muscle cells. CD109 absent in both control and irradiated tissues.
CD109 – AlexaFluor647; CD31 – AlexaFluor488

BMP3

In the AVM tissues, no signal was observed through the endothelium on days 1, 3 and 7 in both irradiated and control tissues. The AVM tissues showed increased intensity in the irradiated tissues on the endothelium on day 21 after irradiation. Comparatively increased intensity was observed in the smooth muscle cells as well to a certain extent. No signal was observed again on day 42 after irradiation in both control and irradiated tissues (Fig. 2.6).

In the CCA, a similar pattern was observed as in the AVM. No signal was observed on days 1, 3 and 7 in either irradiated or control tissues. Increased fluorescence intensity of BMP3 was observed in the endothelium on day 21 in the irradiated tissues. On day 42, no signal was observed in the irradiated tissues as in the controls (Fig. 2.7).

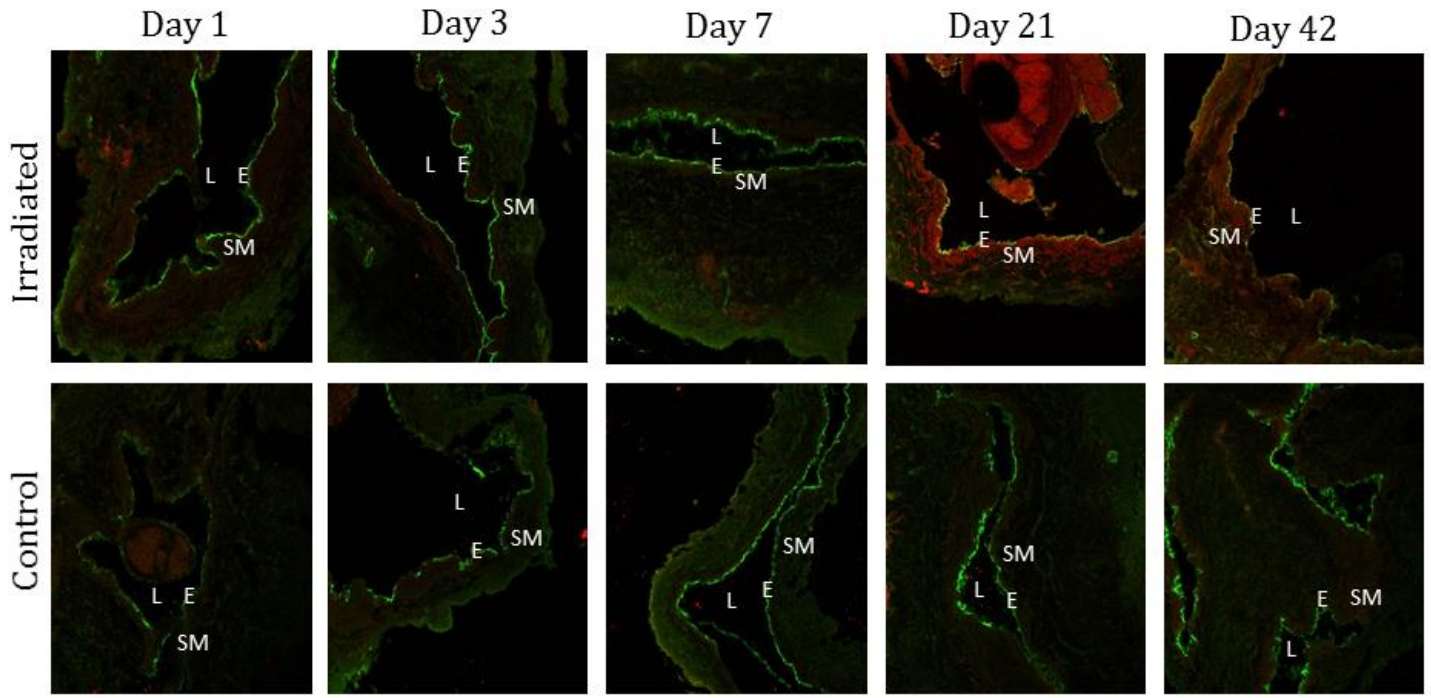

Figure 2.6: BMP3 signal intensity in irradiated and control AVM tissues
L – lumen, E – endothelium, SM – smooth muscle cells. BMP present in day 21 AVM tissues post-irradiation
BMP3 – AlexaFluor647; CD31 – AlexaFluor488

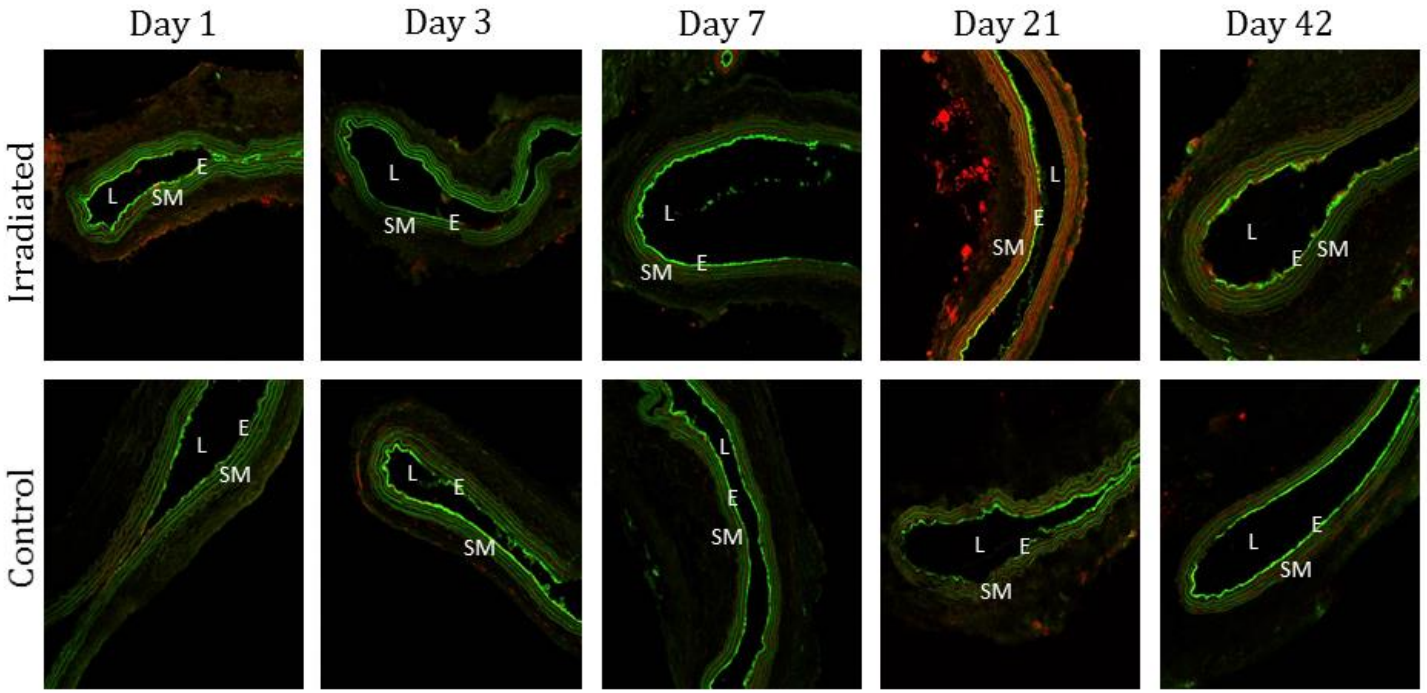

Figure 2.7: BMP3 signal intensity in irradiated and control CCA tissues
L – lumen, E – endothelium, SM – smooth muscle cells. BMP present in day 21 CCA tissues post-irradiation
BMP3 – AlexaFluor647; CD31 – AlexaFluor488

Discussion

Although previous work had identified a number of potential protein markers for the targeting of the AVM vasculature, those efforts had relied largely on *in vitro* work. For further evaluation of these targets, it is necessary to determine if their relative abundance within AVM vasculature is altered in animal tissues after *in vivo* manipulation. To that end, it was examined if irradiation of surgically constructed AVM anastomoses in a rat model affected the relative abundance of CD51, CD109 and BMP3 proteins.

The main findings of the current *in vivo* work indicate that CD51 and BMP3 proteins were present in the endothelial cells of the irradiated AVM and CCA tissues, which is in agreement with the *in vitro* data. CD109 however was absent in both AVM and CCA tissues.

CD51

Integrins are transmembrane proteins that are involved in a variety of cell functions such as adhesion, apoptosis, invasion, migration and survival [35, 36]. They have been reported to show increased expression in certain cancer types, cardiovascular conditions, multiple sclerosis and Crohn's disease [35, 36]. Integrins are heterodimers consisting of α and β subunits[37, 38]. CD51, a target used in this study consists of integrin αv (CD51) chain [38].

The association of integrin proteins with angiogenesis makes them attractive in the field of cancer biology [39-41]. Increased expressions of CD51 have been reported in proliferating endothelial cells and tumours associated with the colon, brain, breast, prostate and pancreas [41-47]. Weak expression is reported in normal vascular endothelial cells [48, 49]. The expression of CD51 in endothelial cells, which is exposed to the blood stream, makes it a potential vascular target in cancer treatment. For instance, in an animal model study by Bieker *et al*, thrombosis was successfully caused in the endothelium of solid tumours by targeting CD51 [50].

For this reason, CD51 was selected for investigating its possible use in AVM vascular targeting. Furthermore, analysis of microparticles in the blood of AVM patients described in Chapter 3 of this thesis indicated that the CD51 antigen was elevated relative to controls.

The data on the relative abundance of CD51 antigen in irradiated AVMs in the rat model contrasts with other findings reported in the literature. In a study conducted on surgically resected tissues from AVMs and cavernous malformations, CD51 expressions in the former was lower than the latter [51]. Increased expression of CD51 was observed in proliferating endothelial cells whereas minimal expression was observed in normal, resting blood vessels [47-49]. The high-pressure blood flow in the AVMs of the non-irradiated rats used in this study could be the reason for the increased relative abundance of CD51 in the CCA tissues. This is not consistent with the EJV tissues where there is decreased relative abundance of CD51 throughout the cohort of non-irradiated animal tissues.

Increased expression of CD51 was reported in human umbilical vein endothelial cells, non-small cell lung cancer and Hela cell lines post irradiation [52, 53]. No study has been reported *in vivo* on the correlation of CD51 with irradiation treatments. In this study, increased relative abundance of CD51 was observed in AVM tissues on days 1 and 3, which diminished by day 42. It is unlikely that this is caused by the apoptosis of cells that are induced by the radiation, as similar patterns would be evident in the CCA as well. Fluorescent intensity levels remain relatively low in the control samples. Interestingly, days 7 and 42 show an increase in fluorescence intensity levels. Another concern here is the presence of CD51 in the non-irradiated (control) samples of the CCA, which was not observed in the AVM samples. This is a concern because the presence of CD51 in non-irradiated tissues is contradictory to the *in vitro* data, where increased relative abundance was observed in radiated samples. Additionally, if CD51 is present in both irradiated and non-irradiated samples, it may not be an attractive molecule for AVM vascular targeting.

CD109

CD109, also known as transforming growth factor β1 (TGF-β1) binding protein is a membrane glycoprotein that acts as a co-receptor to TGF-β1 [54-58]. TGF-β1 belongs to the TGF-β family of cytokines [54, 59]. The TGF-β1 protein is associated with cellular functions such as growth, migration, adhesion, immunity and angiogenesis and is involved in the TGF-β pathway [60-62]. TGF-β signalling is associated with malignancy, wound healing, fibrosis and autoimmune and cardiovascular diseases [63-66]. Increased expressions of TGF-β have been reported in chronic hepatitis and glomerulosclerosis [63, 67, 68]. This signalling pathway can be induced by radiation for potential treatment of solid tumours and has also been reported to be associated with intestinal injury. Studies on targeting the pathway for cancer treatment are underway [69, 70].

Increased expression of CD109 has been reported in rat liver and in animal models of lung post-irradiation as a result of injury [66, 71, 72]. In the current study, an abundance of CD109 in irradiated AVMs was not observed. Other than the one animal from the day 1 AVM cohort, no signal was observed throughout. Since the signal intensity of CD109 was observed in only one of the rats, further investigation is needed to investigate this pattern of expression.

Previous work done in this laboratory had shown that the abundance of the CD109 antigen is increased in irradiated cultured mouse brain endothelial cells exposed to irradiation [32]. However, in work described in Chapter 4 of this thesis, the relative abundance of CD109 was decreased in irradiated biotin-labelled human cerebral microvascular endothelial cells. This was observed in both 10 Gy and 20 Gy treatments.

In a flow cytometry assay using human umbilical vein endothelial cells, CD109 labelled events were only at the limit of detection, suggesting it could be a low abundance protein [73]. This limitation could account for the challenges of detecting it in tissues from *in vivo* experiments. A similar observation was observed by Dong *et al*. Immunohistochemical analysis on tissues resected from

capillary haemangiomas and lobular capillary haemangiomas showed no staining of CD109 just as it was observed in control skin tissues [59].

In a study on TGF-β signalling in vascular dysfunction, decreased expression of endoglin (CD105) leading to decreased expression of TGF-β1 was observed in hereditary haemorrhagic telangiectasia (HHT), which is a genetic AVM condition [58, 74]. The expression of CD105 has not been investigated here in the animal model. However, CD105 levels were investigated in plasma samples (microparticles) from AVM patients in a flow cytometry assay (Chapter 3). Although statistically not significant, CD105 showed decreased levels in AVM patients as compared to healthy volunteers. The decreased expressions of endoglin have downstream effects such as decreased expression of TGF-β1 as well as CD109. This may explain the observations made in this study regarding the absence of CD109 signals in all tissues.

BMP3

Like CD109, BMP3, a member of the BMP subfamily is also part of the TGF-β protein superfamily [75, 76]. BMPs, although known initially for their critical role in bone formation are now widely researched in tumours [75]. BMP4 has been reported for increased expression in colorectal cancer [77]. However, decreased expression of BMP3 was reported in colorectal cancer suggesting its potential role in screening tests for early detection of colorectal cancer [76, 78-80].

BMPs are associated with vascular disorders such as pulmonary arterial hypertension, HHT and atherosclerosis [81]. Further knowledge on the BMP pathway and its signalling is hoped to pave ways for new therapeutics for such conditions [81]. Increased expressions of BMP9 and BMP10 are observed in endothelial cells of HHT patients [81-83]. To the best of the author's knowledge, the correlation of BMP3 to sporadic AVMs has not been reported in the literature.

BMP3 was selected for the current study as previous work in this laboratory identified it as a protein target of radiation treatment of mouse brain endothelial cells [32]. It is noteworthy that BMP3 was not detected in the *in vitro* study carried out in human cerebral microvascular endothelial cells exposed to irradiation (Chapter 4).

In a study on musculoskeletal tissues from human femoral diaphysis, the irradiated samples showed decreased expression of BMP2 and BMP7 [84]. To the best of the author's knowledge, the expression of BMP3 after irradiation has not been reported. The increased fluorescent of BMP3 on day 21 after irradiation is an interesting finding. This is because this study focuses on finding a target molecule that is localised to irradiated tissues for future applications in vascular targeting. Expression patterns in both CCA and AVM tissues are consistent in both irradiated and control groups.

Expression of BMP2 in human aortic vascular smooth muscle cells has been correlated with vascular injury [85]. Increased expression of BMP2 and BMP4 was observed in vascular smooth muscle cells in an immunohistochemical analysis on atherosclerotic vessels [86]. Another study reported BMP4 expression being induced by shear stress in vascular smooth muscle cells. There is no report of BMP3 expression in vascular smooth muscle cells associated with irradiation [87]. In this study, the AVM tissues from day 21 show increased fluorescent intensity on the smooth muscle cells in the irradiated tissues. Endothelial cells that have been irradiated affect the migration and proliferation of smooth muscle cells. Irradiation triggers the TGF-β1/TGF-β-RII/Smad3 pathway in vascular smooth muscle cells [88]. This could be the reason for the increased fluorescent intensity of BMP3 in the endothelium, which then influences its presence in the smooth muscle cells.

The limitation of this study is that it only looks at the localisation of the three selected molecules on the endothelium after irradiation. The level of expression has not been quantified. Semi-quantifying the levels of expression using Image J

software will not be precise because of the co-localisation and also because of the smooth muscle cells that are juxtaposed with the endothelium.

In summary, of the three proteins that were investigated in this study (CD51, CD109 and BMP3), the altered abundance of CD51 and BMP3 antigens after radiation treatment indicates that they may be useful for the targeting of AVMs. These data provide a platform for further detailed investigations of their potential in vascular targeting.

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Chapter 3

Circulating endothelial microparticles in patients with cerebral arteriovenous malformation

Preface to Chapter 3

In Chapter 2, the localisation of CD51, CD109 and BMP3 in the endothelium was investigated in an animal model of AVM post-irradiation and compared to the endothelium of non-irradiated control AVM tissues. In the following chapter, endothelial molecular changes will be explored in humans with AVMs. It is not possible for AVMs to be resected after radiation in humans to study the molecular changes in the endothelium. Therefore, plasma samples from AVM patients will be investigated for the relative abundance of CD51 and CD105 in endothelial microparticles.

The work described in this chapter was presented at the following conferences,

Amal Raj J, Nair S, Combes V, Stoodley M, Davidson A**.** Evaluation of circulating endothelial microparticles in patients with arteriovenous malformations. Poster presented at the 'Australasian Neuroscience Society' Conference, Adelaide, Australia, January 2014

Amal Raj J, Davidson A, Molloy M, Nair S, Stoodley M. Understanding the molecular changes in human brain arteriovenous malformations using circulating endothelial microparticles. Poster presented at the 'Proteomics and beyond' Symposium, Sydney, Australia, November 2012

Abstract

Object. The development of targeted biological treatments for brain arteriovenous malformations (AVMs) requires a better understanding of their endothelial molecular biology, but this is difficult to study in human patients. Endothelial microparticles (EMPs) are a heterogeneous population of circulating particles that are released during endothelial activation or apoptosis. EMPs are known to be markers of vascular injury and endothelial cell disturbance and could potentially be used as indicators of AVM endothelial molecular characteristics. In this study, EMP levels were evaluated in AVM patients and healthy volunteers.

Methods. Blood samples were taken from 9 AVM patients and 14 healthy volunteers. Integrin V-alpha (CD51) and endoglin (CD105) were used as endothelial markers for flow cytometric analysis of the samples.

Results. CD51-labeled and CD105-labeled EMP counts were not significantly different in AVM patients and controls.

Conclusions. Although the EMP counts were not elevated in AVM patients, there remains a potential for their use as indicators of *in vivo* endothelial molecular characteristics.

Introduction

Arteriovenous malformations (AVMs) are the most common cause of brain hemorrhage in children and young adults [1,2]. Currently, AVMs can be treated by surgery, radiosurgery or embolisation. Surgery gives immediate protection from brain haemorrhage but may carry a high perioperative risk of death or disability, especially for large and deep AVMs [3]. Radiosurgery takes 2 – 3 years to obliterate an AVM, during which a haemorrhage can still occur. It can also treat only small AVMs effectively and even in lesions less than 3 cm diameter, the occlusion rate is less than 80% [3-5]. Endovascular embolisation that is intended to reduce the size of an AVM nidus is usually administered in combination with surgery or radiosurgery [6]. A large subset of AVMs remains untreatable with currently available techniques [7]. There is, therefore, a need to find new treatment approaches for AVMs.

Vascular targeting where ligands are developed for effective targeted drug delivery is considered to be a potential molecular treatment for large AVMs [8]. The molecular biology of AVMs is being studied for the development of such new molecular based treatments. These studies are hampered by the difficulty of studying *in vivo* endothelial characteristics in AVM patients. There is a potential to obtain an insight into *in vivo* molecular changes by studying the anucleoid vesicles, called endothelial microparticles (EMPs) that are shed from endothelial cells into the circulation.

EMPs are submicroscopic membranous vesicles that are less than 1 μm in diameter and are very similar to circulating microparticles shed from other cells such as platelets, leukocytes and erythrocytes [9,10]. When stimulated by an activating agent or during apoptosis, the endothelial cell membrane forms a bleb that pinches off into the plasma as a free EMP carrying proteins and phospholipids from the parent cell [11-13]. EMPs are therefore a potential window into the molecular characteristics of the parent cells.

There has been a growing interest in EMP analysis with respect to various clinical conditions in the last decade [11]. EMP levels have been reported as

being elevated in coronary artery disease, postprandial hyperlipidaemia, thrombotic thrombocytopenic purpura, preeclampsia, diabetes mellitus, hypertension, multiple sclerosis, sickle cell disease, paroxysmal nocturnal hemoglobinuria, psoriasis, antiphospholipid antibody syndrome, cerebral malaria and traumatic brain injury [10,11,14-31]. In addition to the potential use of EMPs for studying *in vivo* endothelial molecular characteristics, we hypothesize that the high flow through AVMs causes increased shear stress on the AVM endothelium and an increase in EMP numbers. It is our hypothesis that EMPs can be used as a potential screening tool for the diagnosis of AVMs.

There has been no published work on AVMs with respect to EMPs. The aim of this preliminary study was to determine whether the EMP counts of AVM patients and healthy volunteers differed. Future studies will investigate the feasibility of analysing the molecular characteristics of EMPs as an indicator of the characteristics of the parent endothelial cells.

Materials and methods

Study population

The study was approved by the Macquarie University Human Ethics Committee. Following written informed consent, AVM patients and healthy volunteers were recruited for the study. The participants were 23 – 62 years of age. Healthy volunteers were matched by age and gender to AVM patients. An exclusion criterion was AVM-related hemorrhage within 30 days of presentation.

Preparation of microparticle enriched plasma fractions

Blood samples were taken prior to surgery from AVM patients and at a similar time of the day from healthy volunteers. Blood samples were collected (13 ml) using a 21 G needle. The first 3 ml was discarded to avoid contamination from EMPs due to vascular injury. The tourniquet was left no longer than a minute to avoid frank endothelial damage and activation of EMPs. Acid citrate dextrose (ACD) tubes were used for the collection of blood samples, which were processed within 30 min of collection. The samples were centrifuged at 1550 g for 20 min at room temperature. The upper part of the plasma was removed without disturbing the white buffy coat layer. The resulting platelet poor plasma was centrifuged at 15000 g for 5 min at room temperature. The upper part of the plasma was removed and the resulting platelet free plasma was centrifuged again at 15000 g for 5 min at room temperature. Flow cytometric analysis of the samples was carried out on the same day of collection.

The protocol used in this study was followed to allow for subsequent proteomic studies of the EMP fractions. ACD tubes were used for blood collection because EMP preparation protocols involving heparin and standard citrate were not considered good anti-coagulants for preparing samples for proteomic studies.

Detection of endothelial microparticles

A volume of 20 µL of the sample was singly labelled with annexin V-FITC, CD51-FITC and CD105-PE and incubated for 30 min at room temperature in darkness. Each staining was performed in triplicate. The samples were diluted with 200 µL of binding buffer (Annexin V-FITC) and 200 µL of PBS (CD51 and CD105) immediately before flow cytometry.

EMPs were detected using the Cytomics FC500 flow cytometer (Beckman Coulter). The forward scatter (FS) and side scatter (SS) were set in logarithmic scale and the threshold set at the side scatter parameter. Size calibration flow count beads (10 μm) were used to calculate the flow rate and the samples were analysed over 60 seconds at a flow rate of 55 μL/min. Microparticles were defined as events that were ≤1 μm using calibrated fluorescent beads. Specific labelling with either Annexin-V or antibodies against CD 51–FITC and CD 105–PE were used to further discriminate the total microparticle count and EMP count, respectively (Figures 3.1 – 3.3).

Event counts were used in Mann-Whitney test to determine statistical significance between the two groups.

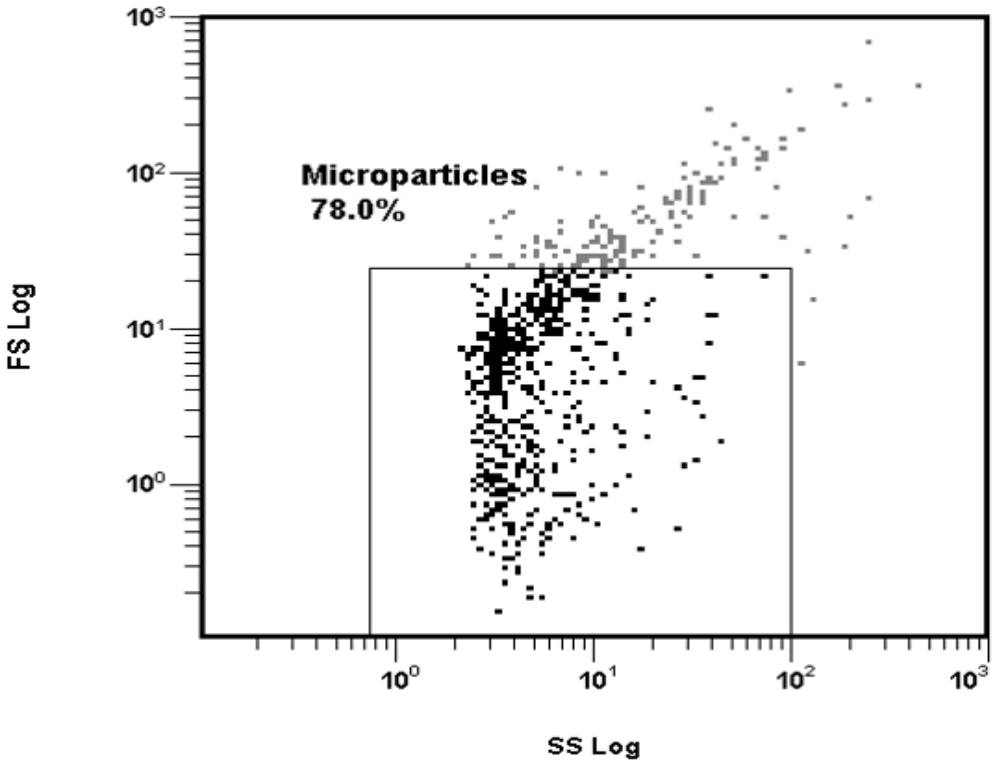

Figure 3.1: Example of a flow cytometry profile of microparticle preparation from healthy volunteers and AVM patients
The box shows the gate used to delineate the microparticle population used for subsequent analysis. The sample was stained with annexin V because it is a natural ligand for phosphotidylserine, which is usually present in the external surface of microparticles.

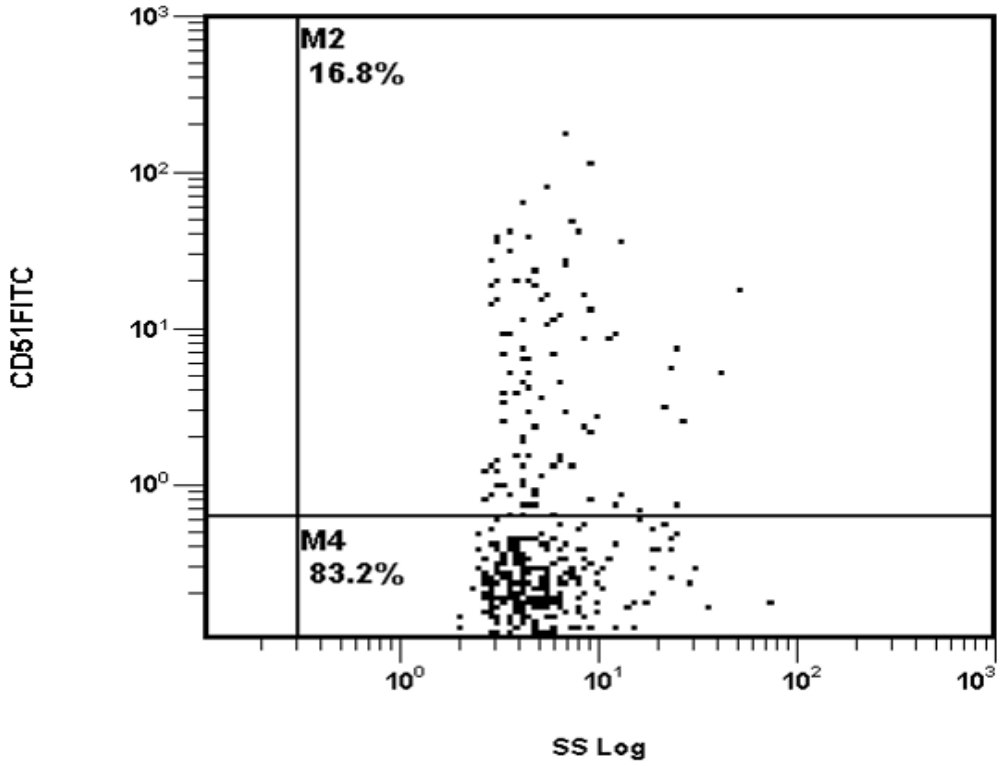

Figure 3.2: Plasma CD51 EMP counts
Axis indicating fluorescent and scatter boundaries where events in the M2 quadrant represent EMPs present in the samples.

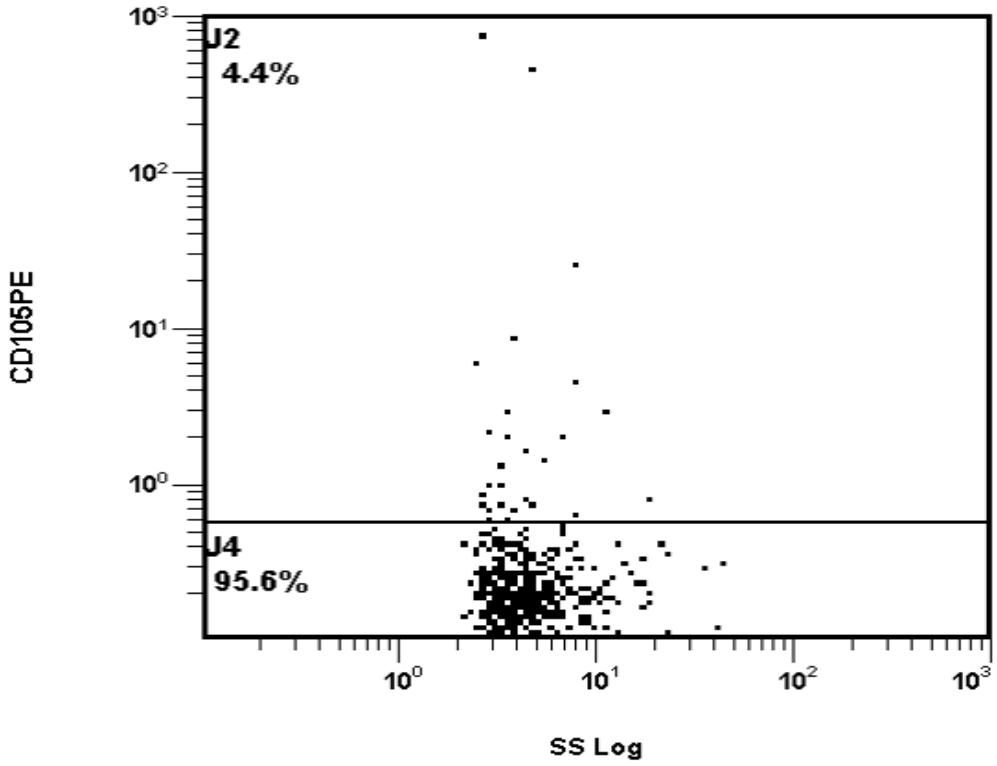

Figure 3.3: Plasma CD105 EMP counts
Axis indicating fluorescent and scatter boundaries where events in the J2 quadrant represent EMPs present in the samples.

Results

Nine AVM patients and 14 healthy volunteers participated in the study. The median age of the participants was 38. The average of EMP total events is shown in Figure 3.4. These differences in CD51 and CD105 counts were not statistically significantly different between AVM patients and controls (CD51: p value = 0.2077, CD105: p value = 0.4122). The large standard error bar seen in the CD105 labeled EMP events could be attributed to one outlier (Figure 3.4).

Because EMP counts were not normally distributed, comparisons were performed using the Mann-Whitney U test. In CD51+ events, the values were $p > 0.05$ (Obtained U value = 63.5, Critical U value = 31) and in CD105+ events, $p > 0.05$ (Obtained U value = 55, Critical U value = 31).

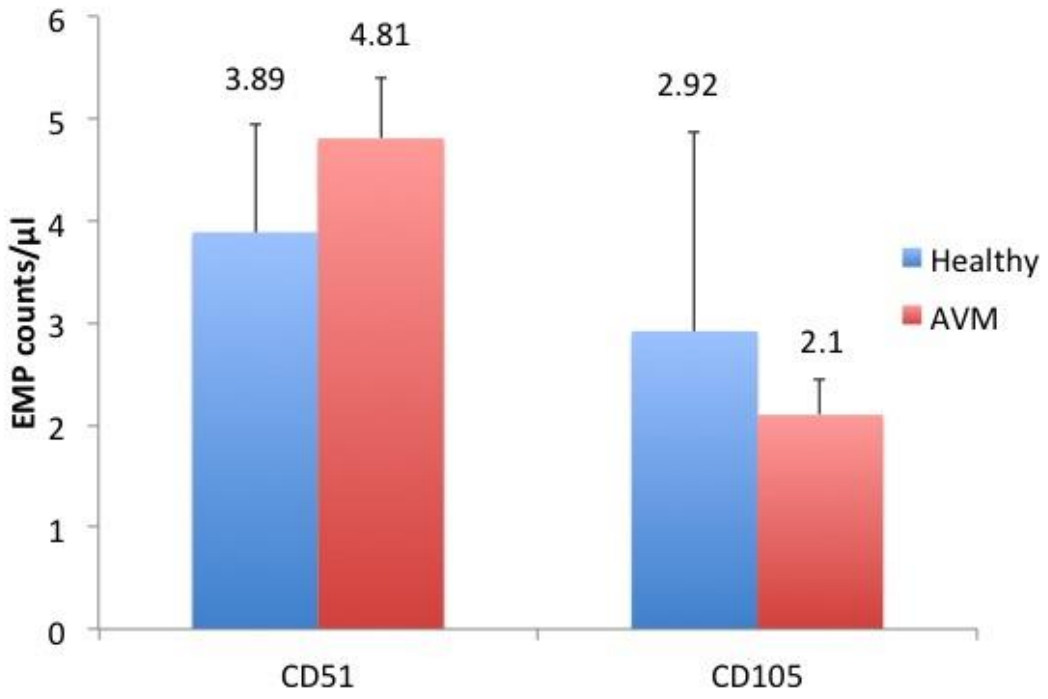

Figure 3.4: EMP counts in blood samples of healthy volunteers and AVM patients
The differences are not statistically significant (CD51: p value = 0.2077, CD105: p value = 0.4122).

Surgery was the recommended mode of treatment for all the AVM patients who participated in this study. The size of the AVM in those patients ranged from

3 mm to 47 mm (median = 19 mm). The EMP levels in the AVM patients related to AVM size are shown in Figure 3.5. There was a correlation between AVM size and EMP count for the CD51 marker (R^2 = 0.5) and no correlation between AVM size and EMP count (CD105: R^2 = 0.1 and Annexin V: R^2 = 0.02).

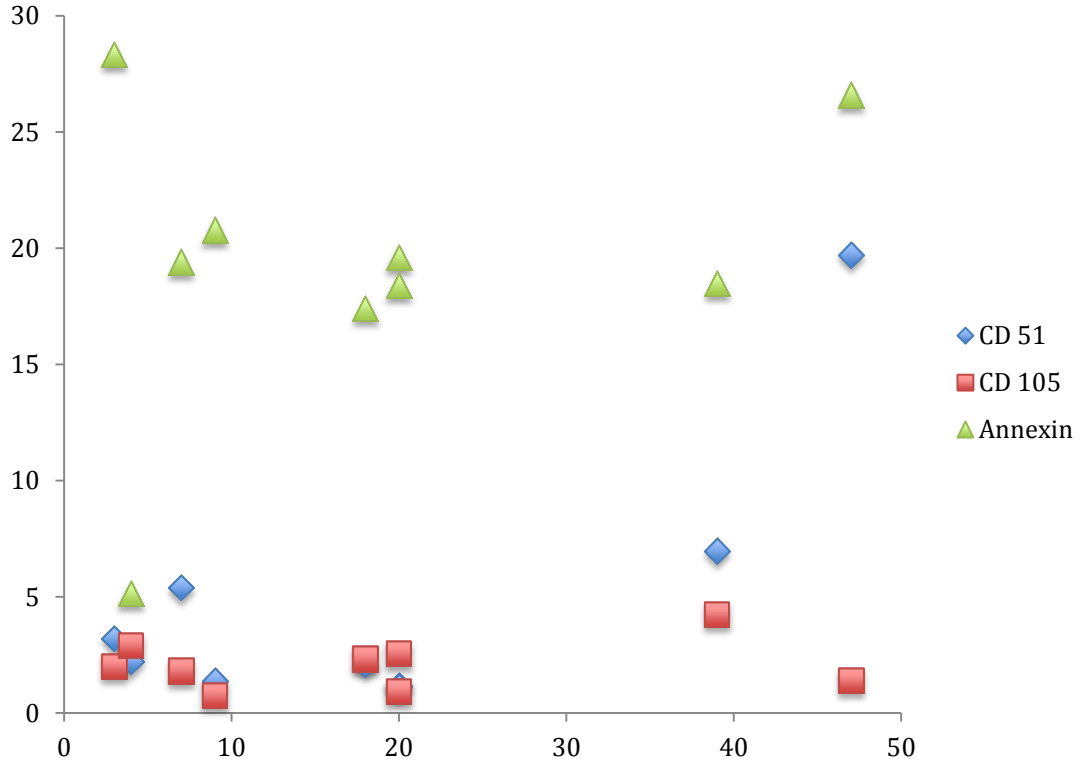

Figure 3.5: Scatter plot of EMP counts and AVM size
(CD51: R^2 = 0.5; CD105: R^2 = 0.05; Annexin V: R^2 = 0.08)

Discussion

AVMs cause intracranial hemorrhage, which may lead to morbidity and mortality [32]. Surgery, embolisation and radiosurgery, which are the current treatment options, are unsuitable for 90% of high grade (IV-V) AVMs [1,33,34]. There is an urgent need of a new treatment for such AVMs.

We are currently investigating a novel treatment where an effector molecule is attached to a ligand and injected systemically [8,35]. Intravascular thrombosis is induced if the ligand predominantly attaches to the AVM vessel. This is being used in cancer therapy for its ability to create tissue necrosis [35].

A successful vascular target must discriminate AVM vessels from normal vessels. We propose radiosurgery as a 'priming' technique where AVM endothelial cells will be selectively altered without any effect on the surrounding tissues [36-38]. Our interest is therefore in determining the endothelial molecular changes that occur after radiation.

The endothelial cell phenotype is typically studied by resection of tissue from patients or animal models. Endothelial microparticles are of particular interest because they offer a potential window into *in vivo* endothelial characteristics without requiring tissue resection or other invasive techniques. Microparticles pinch off from their parent cell during activation or apoptosis and are released into the plasma carrying information about the status of their parent cell [39,40]. Microparticles are currently being exploited for diagnosis and investigation in various clinical conditions [40]. There have been no *in vivo* studies of microparticles in AVMs. This study was aimed at determining the feasibility of EMP detection from humans with AVMs. Before studying if the EMP population was highly altered post radiosurgery, it was important to investigate the levels of EMPs in AVM patients and healthy volunteers to see if there was a specific population of EMPs being released in AVM patients.

The analysis of EMP levels based on the size of the AVM was done to see if an endothelial injury was increased in larger AVMs, which could possibly result in

increased levels of EMP release. We did not find an association between AVM size and EMP count (Figure 3.5).

EMPs are heterogeneous [41]. They carry on their surface phosphatidylserine and several proteins from their parent cells [40]. We have presented the total EMP count based on the two markers, CD51 (integrin V alpha) and CD105 (endoglin). This may not have detected the total population of EMPs present in the sample analysed [41,42]. The sample was put through repeated centrifugation to reduce platelet contamination for subsequent proteomic analysis. The repeated centrifugation may have affected the microparticle count [43],[44]. Because the total number of EMPs detected depends on the marker used, there is a possibility that these two proteins were less in concentration when compared to other proteins. CD105 was selected as one of the markers because increased levels of endoglin have been reported in immunohistochemical studies carried out on AVM tissues [45,46].

The association between EMPs and inflammation has been raised in the past by researchers [14,22,23,47]. It will be interesting to study the inflammatory effect of EMPs on endothelial cells in AVM patients. EMP count is reported to be elevated in vascular conditions such as lupus anticoagulant (2 fold) [47], thrombotic thrombocytopenic purpura, acute coronary syndrome (2.5 fold) and traumatic brain injury [23]. The markers used in those studies include $CD31^{+}/CD42^{-}$, CD62 and CD51 [23,47]. We used $CD31^{+}/CD42^{-}$, CD144 and CD62 in our pilot studies before selecting CD51 and CD105, which were the most effective in capturing EMPs. However, all the pathologic conditions where elevated levels of EMP have been described and correlated with the severity of the disease were systemic diseases where the activation of the endothelium was likely to be extended, while in AVM patients there is a very localised lesion that is extremely small compared to the total vasculature. It is possible that EMPs released by the endothelial cells of the AVM were diluted in the pool of EMPs shed by all the normal cells and could not be differentiated.

Despite our exclusion criteria and our efforts in maintaining the same experimental conditions across the samples, there are some factors that have an effect on the level of circulating microparticles over which we have no control. A high fat meal, reduced physical activity or disturbed sleep contribute to increased levels of EMPs [48-50]. These are parameters that were not possible to control in this study. This may also be the reason for the large standard errors. The large standard error in the CD105 labeled EMP events in healthy volunteers was caused due to an outlier in that data set. At this stage we do not have any reason to exclude it from our analysis (Figure 3.4).

Our goal in this study is to detect a potential molecular target for AVM treatment. As a first step, we analysed the EMP levels in AVM patients and healthy volunteers to see if there was a specific EMP population released in AVM patients. From the obtained results, even though we can conclude that EMPs studied in the present paper are not markers for the presence of an AVM, the discovery of a potential vascular target may still be possible. This can be carried out by proteomic analysis of the EMP samples. The proteomic signature of the microparticles in an AVM patient may be different to that of a non-AVM subject. If a specific proteomic profile is found in the AVM patients, that could indicate a potential vascular target for the treatment of AVMs.

Conclusion

The primary objective of this study was to compare the EMP levels between AVM and non-AVM subjects. The levels of EMP are similar in both AVM and non-AVM subjects. Proteomic study of the microparticles between the two groups may give us an insight into the proteome of the AVM patients with a specific profile, which gives us the hope of finding a potential vascular target for the effective treatment of large AVMs.

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Addendum

The protein targets that were evaluated in the animal study in Chapter 2 was based on an earlier *in vitro* study of irradiated mouse brain endothelial cells. To obtain a clearer idea of proteins that may be elevated in AVM patients, it was decided to study the EMP proteomic signature of AVM patient plasma samples. Of all the microparticles, those that are of endothelial origin are the least abundant. This made it very challenging to sort endothelial microparticles through flow cytometry assays for further proteomic assays. Instead, mass spectrometric analysis was attempted on microparticle preparations obtained from the plasma of AVM patients and healthy volunteers. A microparticle pellet was prepared through repeated centrifugations at high speed. The pellet was digested with trypsin and subsequently analysed by iTRAQ to explore the proteomic signatures of the AVM and control samples. Unfortunately, only plasma proteins such as actin, lipopolysaccharide binding protein and heparin cofactor 2 were identified. None of the proteins detected were of endothelial cell origin. Decreasing the centrifugation speeds resulted in increased platelet contamination and increasing the centrifugation resulted in increased plasma proteins to be detected. After several attempts to resolve technical difficulties, it was decided to not proceed with this study. A similar study was planned in rats with AVM anastomosis. The animals were irradiated and blood samples were collected at different time points to investigate the EMP levels and proteomic signatures from plasma samples collected post-irradiation. Due to similar difficulties as with human samples, the microparticle proteomic study in rats was terminated.

Extensive consultation with experts in the field indicated that the best approach to overcome the limitations experienced in this study can only be overcome with larger sample sizes. This is currently underway and a large cohort of samples has been collected from AVM patients in India. This is an ongoing project.

Chapter 4

Identification of radiation–induced membrane proteins in human cerebral microvascular endothelial cells for the treatment of brain arteriovenous malformations

Preface to Chapter 4

In Chapter 3, after detection of endothelial microparticles in plasma samples from AVM patients, proteomic analysis in microparticles was attempted in plasma samples from animal models and human AVM patients to obtain a clearer picture of the proteomic signature in AVM patients and also to investigate the proteomic signature post-irradiation. The project was discontinued due to technical issues. In the following chapter, the radiation-induced proteomic signature will be investigated in human brain microvascular endothelial cells (D3 cell line).

Abstract

Background: Intracerebral arteriovenous malformations (AVMs) cause brain haemorrhage, often leading to death or permanent disability. Over one third of AVMs that are large or located in critical regions of the brain cannot be cured with existing treatment options. There is a need for a novel molecular based treatment to treat such AVMs. The aim of this study is to find a potential vascular target. Priming the endothelium with radiation induces molecular changes, which can then be targeted by ligands attached to effector molecules.

It is hypothesised that radiosurgery induces sufficient molecular changes in endothelial cells to differentiate them from non-irradiated cells.

Methods: Human cerebral microvascular endothelial cells were irradiated with 10 Gy and 20 Gy treatments. Two days post-irradiation, the cells were biotin labeled and analysed by LC/MS/MS.

Results: A total of 168 proteins were detected, 27 of which were significantly more abundant in the 10 Gy treatments, 17 were significantly more abundant in the 20 Gy treatments and 9 were common to both groups. Four proteins were selected for further validation by immunofluorescence and western blot analyses.

In the immunofluorescence assay, talin and myoferlin showed significantly increased fluorescent intensity in 20 Gy compared to controls. HADHA and elongation factor 2 showed no significant difference between the irradiated and control groups.

In the western blot analysis, talin showed increased band intensity in 20 Gy treatments. There was no significant difference observed in myoferlin, HADHA and elongation factor 2. Knowledge about the altered endothelial cell phenotype post-irradiation will contribute to the search for a potential vascular target for the treatment of AVMs.

Introduction

Brain arteriovenous malformations (AVMs) are congenital lesions where the arteries and veins form a tangled web–like connection called a 'nidus' [1-3]. These short circuit connections cause high-pressure blood to flow through the fragile cerebral veins that leads to haemorrhage [2, 4]. Children and young adults are the most commonly affected population [5]. Patients who survive a haemorrhage often live with permanent neurological deficits [6, 7].

Surgical resection is the favoured approach to treating AVMs but is not possible for all lesions depending on size and location of the lesion [8-13]. Stereotactic radiosurgery (SRS) is an attractive alternative, as it is non–invasive and precisely targets lesions with a single, high dose of radiation, without having any effects on the surrounding tissues [14]. There are limitations to the effectiveness of SRS. It takes approximately 2 – 3 years to successfully cause thrombosis in the AVM vessels. Until thrombosis occurs, the patient is still at risk of haemorrhage [14-17]. Lastly, there is no guarantee of successful obliteration after SRS treatment [18]. There is therefore a need for a new treatment for those AVMs that cannot be cured with currently existing treatment options.

In response to this need for a new treatment, it has been proposed that a molecular based treatment for AVMs in combination with radiosurgery could be useful. It has been established that the AVM endothelium is phenotypically different to the endothelium of normal vasculature but is not sufficiently different to enable precise molecular targeting [5, 7, 9, 16-19]. Since radiation causes DNA damage, cell apoptosis, inflammation and endothelial modification, it has been proposed SRS may be employed as a means of altering the protein signatures of those tissues so that the AVM endothelium may be differentiated from the normal endothelium [20-22]. Hence, the overall goal of this project is to identify radiation-induced protein markers that differentiate the AVM vasculature and target those markers with ligands coupled to effectors that promote intravascular thrombosis.

The aim of this study was to explore radiation-induced changes in the expression of proteins in human cerebral microvascular endothelial cells (HCMEC).

Methodology

Cell culture

HCMEC (D3 cell line) were cultured in T75 flasks, 6–well plates and 8–well chamber slides. All culture vessels were coated with rat-tail collagen. The endothelial cells acquired from the D3 cell line were cultured in EBM growth medium (Bioscience, catalogue #CC3156) and were incubated at 37° C in 5% CO_2.

Irradiation

When the cultures were 30% confluent, the cells were irradiated using a 6 MV linear accelerator (LINAC, Elekta Synergy, Crawley, UK) at Macquarie University Hospital, Sydney, Australia. The cells received a dosage of 10 Gy or 20 Gy. Cells in the control samples were not irradiated.

Biotin Labelling

At 48 hours post irradiation, the cells in T75 flasks (n = 12) were washed in four changes of ice–cold PBS. After the final wash, freshly prepared biotin labelling solution (120 μM) (ThermoFisher, catalogue #21335) was added to each of the flasks. The flasks were incubated at room temperature in a rocker for 5 min. The biotin labelling reaction was terminated with the addition of Tris – HCl (pH 7.4; 2 mM final concentration; 5 min). After this, the cells were washed again in four changes of ice-cold PBS. Subsequently, 0.5 mL of cold lysis buffer (NP40 10%, SDS 20%, protease mix 100X (GE healthcare, catalogue #80650123), EDTA 0.5M, PBS 10X, Milli water) was added to each of the flasks. The cells were scraped using a cell scraper, collected into centrifuge tubes and stored at -80° C overnight.

The samples were thawed on ice and sonicated thrice for 15 sec at 40% power, with a 30 – 60 sec interval between each sonication. The cell samples were centrifuged at full speed for 10 min at 4° C to clarify. The supernatant was then collected in fresh centrifuge tubes. Part of the supernatant was used for bicinchoninic acid assay (Pierce Biotechnology Inc.) to determine total protein concentration and the remainder was stored at -80° C for streptavidin enrichment and proteomic analysis.

Streptavidin enrichment and trypsin digestion

The samples were thawed on ice and buffers A (1% NP40, 0.1% SDS, 1X PBS), B (1M NaCl, 0.1% NP40, 1X PBS) and C (digestion buffer 50mM ammonium hydrogen carbonate, 0.5% sodium deoxycholate) were prepared. Streptavidin–sepharose high performance beads (GE healthcare, catalogue #17511301) were washed thrice in buffer A to remove ethanol and impurities. After every wash, the resin was centrifuged at 2000 rpm, resuspended in 400 µL of buffer A and agitated for 30 sec. The wash buffer was removed and the desired amount of protein was added to the resin (1 mg protein to 200 µL resin). The samples were rotated at room temperature for two hours, centrifuged at high speed and supernatant removed. The resin containing biotin–labelled proteins were then washed thrice in buffer A (400 µL), twice in buffer B (400 µL) and eight times in buffer C (400 µL). The resin was resuspended in buffer C (400 µL) and the samples were kept on ice until trypsin digestion. Iadoacetamide was added to alkylate the samples. Trypsin (2 µg) was added to the protein samples and they were left to incubate overnight at 37° C under constant agitation. The samples were centrifuged thrice at 10000 g for 10 min each spin to remove sodium deoxycholate. After removal of the supernatant, the samples were concentrated by lyophilisation (SpeediVac) and then resuspended in 2% acetonitrile for LC/MS/MS analysis.

LC/MS/MS analysis

A TripleTOF 5600 mass spectrometer (AB SCIEX) fit with a NanoLC™ ultra liquid chromatography syste (Eksigent) and a cHiPLC unit (Eksigent) was used for LC/MS/MS analysis. A 200 µm × 0.5 mm nano cHiPLC trap column at a flow rate of 10 µl/minute for 5 min and a 150 mm × 200 µm nano cHiPLC column at a flow rate of 600 nL/minute over 80 min operating a linear gradient from 5% to 45% were used for reverse phase separations.

The protein samples were put through positive ion nano flow analysis utilising ion spray voltage, heater interface temperature, curtain gas flow of 2.5 kV, 150° C, 25 and 16 respectively. A 'top 20' approach that utilises a full MS survey scan was carried out for data dependent acquisition experiments. This was followed by 20 MS/MS product ion scans (100 – 1500 amu, 100 ms each), which

were then collected for ions with a 2+ to 4+ charge-state and an ion intensity threshold of 150 counts per second. LC/MS/MS data files were converted to MASCOT generic files and searched using MASCOT (Matrix Science, UK) against all human entries in the Swissprot database (SwissProt_2016_02, Feb–17, 2016). Search parameters included the use of trypsin as enzyme, a maximum number of missed cleavages of 1, carbidomethyl modification of cysteine and oxidation of methionine, a peptide mass tolerance of 50 ppm and fragment ion tolerance of 0.1 Da and an ion score cut–off of 31. Protein quantitation was performed by uploading MASCOT (.dat) files for each replicate (triplicates for each group, controls, 10 Gy and 20 Gy) into Scaffold (V_4.4.3, Proteome Software Inc, Portland, OR) and using the Normalised Spectral Abundance Factor (NSAF) approach. All treatment replicates were assessed against control samples for fold change (>2 or <0.5) and t–test ($p < 0.05$). Differentially expressed proteins and proteins detected only in all three replicates of treated samples were uploaded to Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City) and the relationships between proteins in a given network assessed. Proteins in a network are represented as nodes and biological relationships as lines. Red nodes represent up regulated proteins and down regulated proteins are represented as green nodes.

Western blotting

At 48 hours post irradiation, total proteins were extracted from cells cultured in 6-well plates using radioimmunoprecipitation assay (RIPA) lysis buffer. The buffer consisted of 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% deoxycholate, 0.1% SDS, 1% Triton-X-100, 0.5 M EDTA (pH 8) and protease inhibitor mix. The samples were then sonicated, centrifuged and the supernatant was collected to determine the total protein concentrations using the BCA assay.

Fifteen micrograms of each protein sample were loaded onto pre–cast SDS–PAGE gels (Life Technologies, catalogue #NP030BOX) and run at 200 V for an hour. The proteins were transferred to nitrocellulose membranes (Bio-Rad) and the membranes were blocked with skim milk for an hour at room temperature.

The membranes were probed with the following primary antibodies: talin (Abcam, catalogue #78921) at a 1:500 dilution, myoferlin at a 1:500 dilution (Abcam, catalogue #76746) trifunctional enzyme sub–unit alpha/ HADHA (ThermoFisher, catalogue #PA5-27348) at a 1:500 dilution and elongation factor 2 (Abcam, catalogue #75748) at a 1:3000 dilution and incubated overnight at 4° C. Peroxidase conjugated rabbit anti-mouse antibodies (Abcam, catalogue #6728) were added for talin and myoferlin and goat anti-rabbit antibodies (Abcam, catalogue #6721) were added for HADHA and EF2 at a dilution of 1:2000 and incubated for an hour at room temperature. The membranes were developed using an ECl kit (Bio-rad, catalogue #1705061) according to manufacturer's instructions and visualised using the Bio–Rad GelDoc. GAPDH (Abcam, catalogue #181602) was used to normalise the expression of the target proteins.

Protein bands from western blots were quantified using Image Lab software. The intensity of the GAPDH bands on the membranes was used to normalise the intensities of the other target protein bands. The relative intensity was calculated using the formula, normalised protein expression = adjusted volume of the protein of interest/ relative quantification of GAPDH.

Immunofluorescence

At 48 hours post–irradiation, the cells cultured in 8–well chamber slides were fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton–X100. The cells were blocked with foetal bovine serum for an hour at room temperature and incubated overnight with the respective primary antibodies at 4° C. Antibodies to talin, myoferlin, trifunctional enzyme sub–unit alpha/ HADHA and elongation factor 2 were added at a 1:100 dilution. Secondary antibodies attached to fluorophores were added at a dilution of 1:400 and incubated for an hour at room temperature. Donkey anti-mouse Alexa Fluor 647 (Thermo Fisher, catalogue #A-31571) was added for talin and myoferlin and donkey anti-rabbit Alexa Fluor 647 (Thermo Fisher, catalogue #A-31573) for HADHA and EF2 respectively. The cell nuclei were stained with Hoechst (5 μg/mL). A Zeiss microscope fitted with an AxioCam HRc camera and Zen 2012 software was used to acquire images after immunofluorescence. The integrated intensity levels

were quantified using Image J, using the formula, normalised density = (raw intensity density – background intensity)/ area. The control and irradiated groups were compared using the unpaired 2-tailed student's t-test. GraphPad Prism 6 was used for statistical analysis.

Results

Mass spectrometry of biotin-labelled cells detected a total of 168 proteins in the control vs. 10 Gy and control vs. 20 Gy experiments. The cut-off p-value for differentially expressed proteins was kept as 0.00030. Out of the 168 proteins, 141 proteins were more abundant and 27 were less abundant in the 10 Gy treatments and 138 proteins were more abundant and 30 less abundant in the 20 Gy treatments. A total of 23 proteins were significantly ($p<0.05$) more abundant and four less abundant in the 10 Gy vs. control data set and 13 proteins were significantly ($p<0.05$) more abundant and four less abundant in the 20 Gy vs. control data set (Table 4.1 & Table 4.2). Out of the nine statistically significant ($p<0.05$) proteins that were common to both data sets, seven were more abundant (Fig. 4.1). In the differentially abundant list of proteins that were statistically significant ($p<0.05$), five were membrane proteins, three were mitochondrial and the remaining 19 were cytoplasmic/cytoskeletal proteins in the 10 Gy treatments. In the 20 Gy treatments, five were membrane proteins, three were mitochondrial and 11 were cytoplasmic/cytoskeletal proteins. The list of proteins was analysed using the Uniprot database for its sub-cellular locations (http://www.uniprot.org).

Network pathways for more abundant proteins and network function scores computed by Ingenuity Pathway Analysis software are represented in Supplementary Figures 4.1 & 4.2 and Supplementary Tables 4.1 & 4.2.

Table 4.1: Statistically significant proteins in 10 Gy treatments

Uniprot Accession	Name	Location	P-value (T-test)	Fold Change
ITB1_HUMAN	Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2	Cell Membrane	0.0005	0.4
ECHA_HUMAN	Trifunctional enzyme subunit alpha, mitochondrial OS=Homo sapiens GN=HADHA PE=1 SV=2	Mitochondria	0.00058	INF
FLNB_HUMAN	Filamin-B OS=Homo sapiens GN=FLNB PE=1 SV=2	Cytoplasm	0.002	INF
EF2_HUMAN	Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4	Cytoplasm	0.0023	12
XRCC5_HUMAN	X-ray repair cross-complementing protein 5 OS=Homo sapiens GN=XRCC5 PE=1 SV=3	Nucleus	0.0023	INF
CTNA1_HUMAN	Catenin alpha-1 OS=Homo sapiens GN=CTNNA1 PE=1 SV=1	Cytoplasm	0.0031	INF
TCPD_HUMAN	T-complex protein 1 subunit delta OS=Homo sapiens GN=CCT4 PE=1 SV=4	Cytoplasm	0.0031	INF
UBR4_HUMAN	E3 ubiquitin-protein ligase UBR4 OS=Homo sapiens GN=UBR4 PE=1 SV=1	Cell Membranre	0.0031	INF
MVP_HUMAN	Major vault protein OS=Homo sapiens GN=MVP PE=1 SV=4	Cytoplasm	0.0078	INF
TLN1_HUMAN	Talin-1 OS=Homo sapiens GN=TLN1 PE=1 SV=3	Cytoplasm	0.0078	INF
RPN2_HUMAN	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 OS=Homo sapiens GN=RPN2 PE=1 SV=3	Endoplasmic reticulum, Membrane	0.0081	0.3
ANXA1_HUMAN	Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	Nucleus, Cytoplasm	0.013	INF
ANXA5_HUMAN	Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2	Cytoplasm	0.013	INF

PTBP1_HUMAN	**Polypyrimidine tract-binding protein 1 OS=Homo sapiens GN=PTBP1 PE=1 SV=1**	**Nucleus**	**0.014**	**3.7**
1B40_HUMAN	HLA class I histocompatibility antigen, B-40 alpha chain OS=Homo sapiens GN=HLA-B PE=1 SV=1	Membrane	0.018	0.2
LMNA_HUMAN	Prelamin-A/C OS=Homo sapiens GN=LMNA PE=1 SV=1	Nucleus	0.018	5.4
RS8_HUMAN	40S ribosomal protein S8 OS=Homo sapiens GN=RPS8 PE=1 SV=2	Cytoplasm	0.021	0.6
ATPB_HUMAN	ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3	Mitochondria	0.026	2.3
SPTN1_HUMAN	Spectrin alpha chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTAN1 PE=1 SV=3	Cytoplasm	0.03	INF
FERM2_HUMAN	Fermitin family homolog 2 OS=Homo sapiens GN=FERMT2 PE=1 SV=1	Cytoplasm	0.034	INF
EFTU_HUMAN	Elongation factor Tu, mitochondrial OS=Homo sapiens GN=TUFM PE=1 SV=2	Mitochondria	0.038	0.1
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK OS=Homo sapiens GN=AHNAK PE=1 SV=2	Nucleus	0.041	INF
RN213_HUMAN	E3 ubiquitin-protein ligase RNF213 OS=Homo sapiens GN=RNF213 PE=1 SV=3	Cytoplasm	0.041	INF
PRKDC_HUMAN	DNA-dependent protein kinase catalytic subunit OS=Homo sapiens GN=PRKDC PE=1 SV=3	Nucleus	0.043	6.5
PLEC_HUMAN	Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3	Cytoplasm	0.044	4.7
RL8_HUMAN	60S ribosomal protein L8 OS=Homo sapiens GN=RPL8 PE=1 SV=2	Cytoplasm	0.045	INF
ACLY_HUMAN	ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=1 SV=3	Cytoplasm	0.048	INF

Table 4.2: Statistically significant proteins in 20 Gy treatments

Uniprot Accession	Name	Location	P-value (T-test)	Fold Change
ITB1_HUMAN	Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2	Cell membrane	**0.0031**	**0.4**
PLAK_HUMAN	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3	Cytoplasm, membrane	**0.0043**	**INF**
ACLY_HUMAN	ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=1 SV=3	Cytoplasm	**0.0069**	**INF**
TLN1_HUMAN	Talin-1 OS=Homo sapiens GN=TLN1 PE=1 SV=3	Cytoplasm	**0.0085**	**INF**
COPG1_HUMAN	Coatomer subunit gamma-1 OS=Homo sapiens GN=COPG1 PE=1 SV=1	Cytoplasm	**0.013**	**INF**
1B40_HUMAN	HLA class I histocompatibility antigen, B-40 alpha chain OS=Homo sapiens GN=HLA-B PE=1 SV=1	Membrane	**0.016**	**0.3**
ANXA1_HUMAN	Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	Nucleus, Cytoplasm	**0.018**	**INF**
FERM2_HUMAN	Fermitin family homolog 2 OS=Homo sapiens GN=FERMT2 PE=1 SV=1	Cytoplasm	**0.019**	**INF**
MUC18_HUMAN	Cell surface glycoprotein MUC18 OS=Homo sapiens GN=MCAM PE=1 SV=2	Membrane	**0.02**	**0.4**
EF2_HUMAN	Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4	Cytoplasm, nucleus	**0.022**	**9**
MVP_HUMAN	Major vault protein OS=Homo sapiens GN=MVP PE=1 SV=4	Cytoplasm	**0.029**	**INF**
SAMH1_HUMAN	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 OS=Homo sapiens GN=SAMHD1 PE=1 SV=2	Nucleus	**0.029**	**7.4**

FAS_HUMAN	**Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3**	**Cytoplasm**	**0.034**	**4.4**
PRDX1_HUMAN	Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1	Cytoplasm	**0.036**	**0.3**
ITA5_HUMAN	Integrin alpha-5 OS=Homo sapiens GN=ITGA5 PE=1 SV=2	Membrane	**0.043**	**0.1**
ATPB_HUMAN	ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3	Mitochondria	**0.047**	**2.2**
SERPH_HUMAN	Serpin H1 OS=Homo sapiens GN=SERPINH1 PE=1 SV=2	Endoplasmic reticulum, Cytoplasm	**0.05**	**3.8**

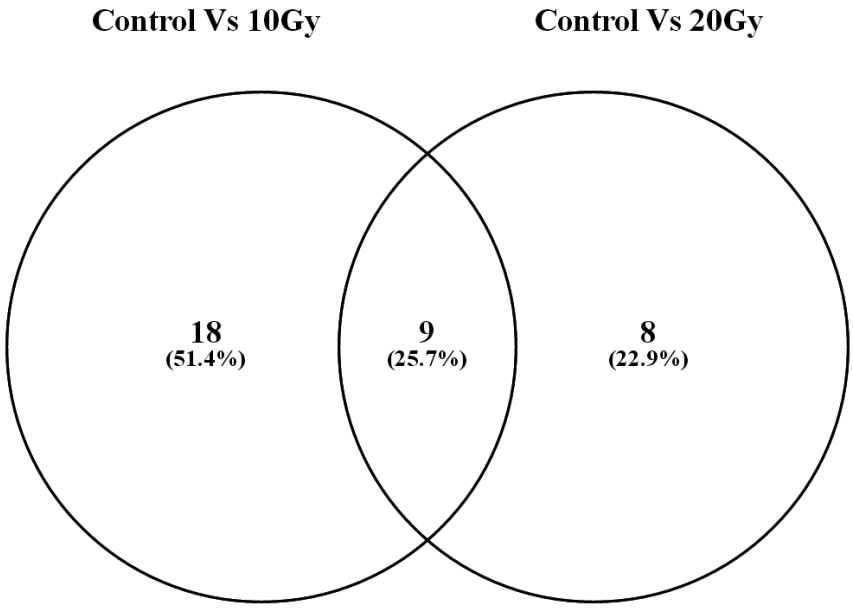

Figure 4.1: Venn diagram representation of statistically significant ($p<0.05$) proteins - Control vs. radiated
Nine common proteins were identified in 'Control Vs 10 Gy' and 'Control Vs 20 Gy'.

Nine proteins were significantly more abundant in all triplicate experiments – integrin beta 1, elongation factor 2, major vault protein, talin, annexin A1, HLA class I histocompatibility antigen, ATP synthase subunit beta, fermitin family homolog 2 and ATP citrate synthase. Out of the nine proteins, we selected talin and elongation factor 2 that were more abundant in both 10 Gy and 20 Gy treatment groups, HADHA that was significantly more abundant in the 10 Gy treatment group and myoferlin for further validation by western blot and immunofluorescence analyses. Talin, HADHA and elongation factor 2 were selected because they were all highly abundant and myoferlin, although it was not statistically significant was selected because it was a membrane protein.

Talin

Increased relative abundance of talin was observed in the radiated samples as compared to the control samples with a p-value of 0.0078 for the control vs. 10 Gy treatments (Table 4.1) and a p-value of 0.0085 for the control vs. 20 Gy treatments (Table 4.2). The differences were dose-dependent. The differences were statistically significant ($p < 0.05$) between control and 20 Gy

radiated samples in western blot analysis. Band intensity levels in 20 Gy samples were higher than in 10 Gy in the western blot analyses (Fig. 4.2). In the immunofluorescence analysis, staining was observed in the cytoplasmic region. The differences between 10 Gy and control samples showed statistical significance ($p<0.05$) in immunofluorescence analyses where the results were semi-quantified using Image J (Figure 4.3).

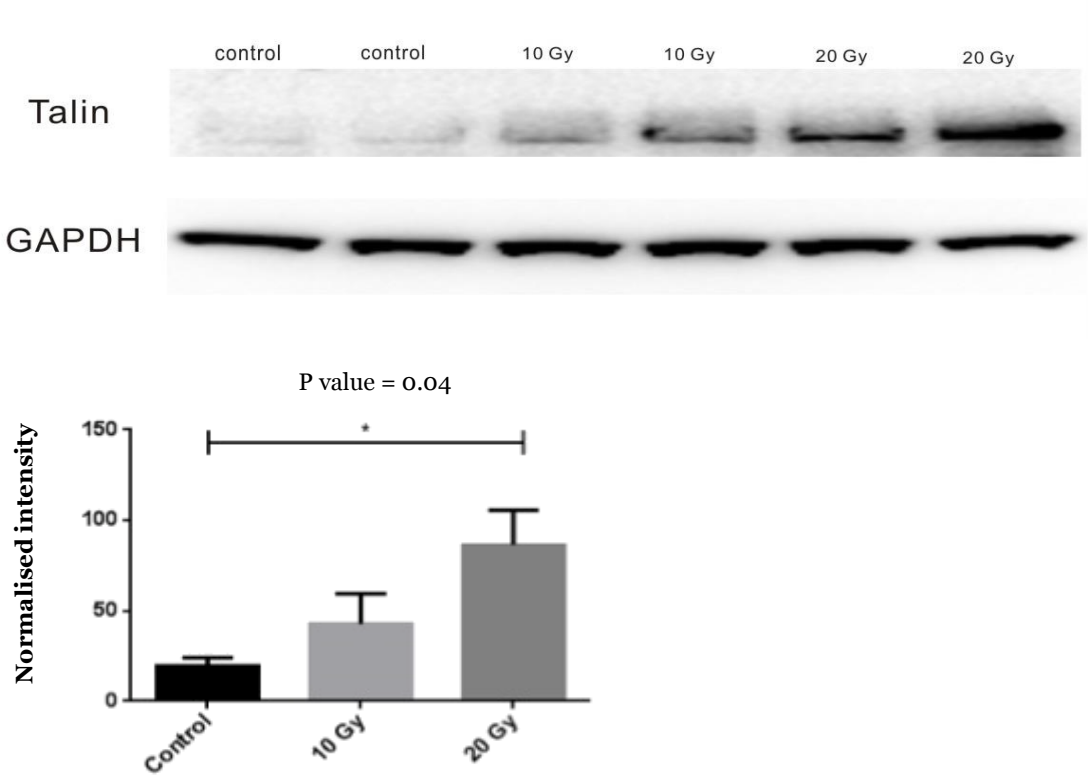

Figure 4.2: Western blot analysis of talin expression from HCMEC
At 48 hours post irradiation, the intensity of the talin bands were normalised against GAPDH band intensity for control, 10 Gy and 20 Gy samples. Talin band intensity levels were significantly higher ($p<0.05$) in 20 Gy than the control and 10 Gy treatments.

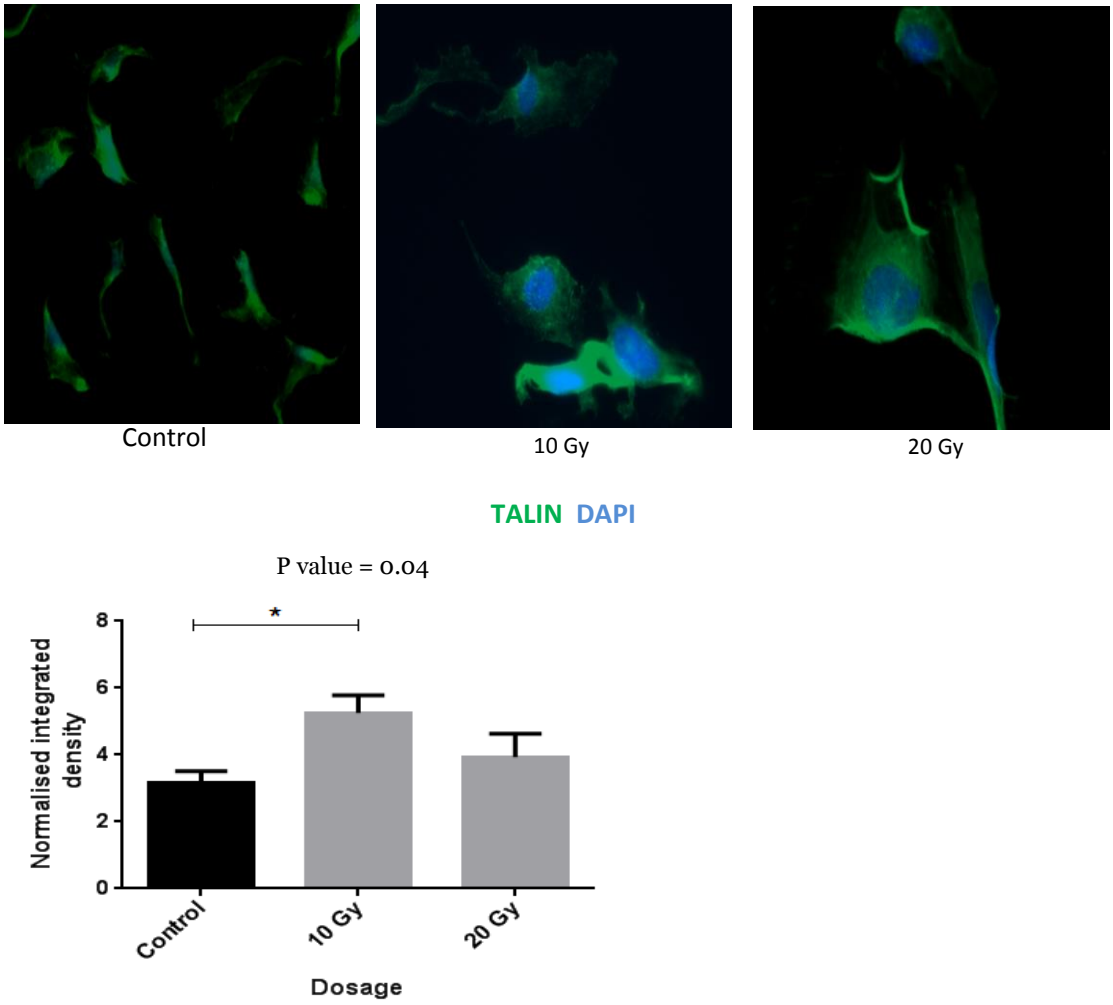

Figure 4.3: Immunofluorescence analysis of talin expression from HCMEC
At 48 hours post irradiation, cells were fixed with paraformaldehyde and the fluorescence intensity (normalised integrated density) due to Talin was determined. Donkey anti-mouse secondary antibodies were attached to Alexa Fluor 647 fluorophores. Significantly increased normalised integrated density ($p<0.05$) was observed in the 10 Gy irradiated samples compared to controls.

Myoferlin

In the LC/MS/MS, myoferlin was not statistically significant in its relative abundance in control vs. 10 Gy and control vs. 20 Gy treatments. Neither was there any difference in band intensities in the western blot analysis ($p = 0.6$) (Fig. 4.4). In the immunofluorescence analysis, myoferlin staining was concentrated more towards the perinuclear region and in the cytoplasm. Increased intensity levels were observed in the radiated samples as compared to the controls (Fig. 4.5). The differences were statistically significant between the radiated and control samples when semi-quantified using Image J ($p = 0.01$).

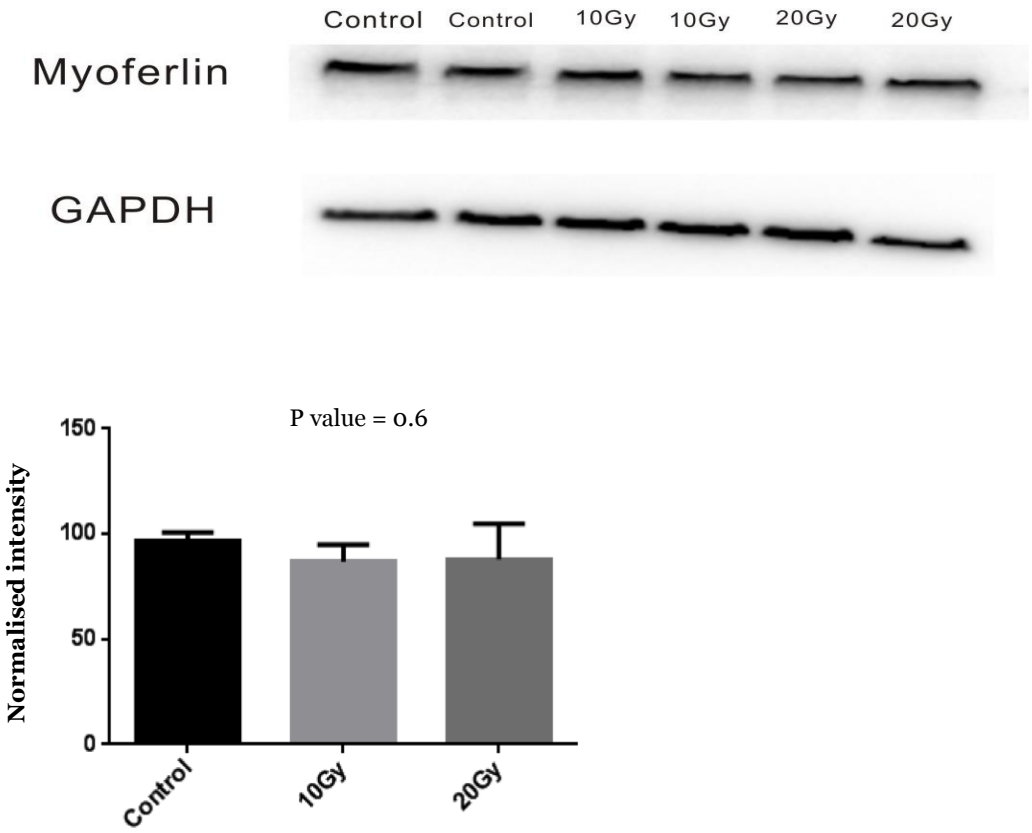

Figure 4.4: Western blot analysis of myoferlin expression from HCMEC
At 48 hours post irradiation, the intensity of the myoferlin bands were normalised against GAPDH band intensity for control, 10 Gy and 20 Gy samples. No significant difference ($p>0.05$) was observed between irradiated and control samples.

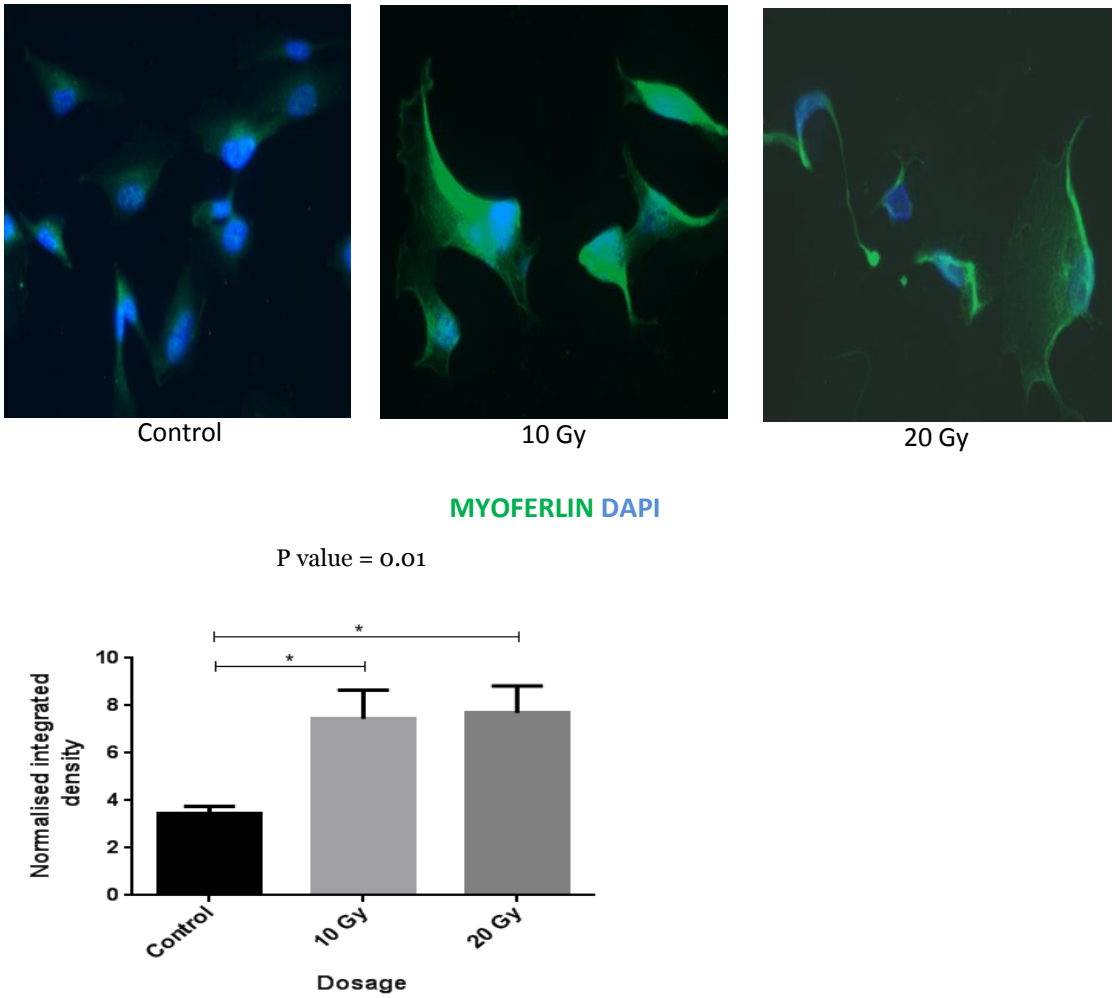

Figure 4.5: Immunofluorescence analysis of myoferlin expression from HCMEC

At 48 hours post irradiation, cells were fixed with paraformaldehyde and the fluorescence intensity (normalised integrated density) due to myoferlin was determined. Donkey anti-mouse secondary antibodies were attached to Alexa Fluor 647 fluorophores. Significantly increased normalised integrated density ($p<0.05$) was observed in irradiated samples compared to controls.

HADHA

Increased relative abundance of HADHA was observed in the radiated samples as compared to the control samples with a p-value of 0.00058 for the control vs. 10 Gy treatments (Table 4.1). No differences were observed between the radiated and control groups in western blot analysis (p = 0.4) (Fig. 4.6). In the immunofluorescence analysis, staining was observed through the cytoplasm (Fig. 4.7). No difference in the intensity levels was observed between the irradiated and control samples (p = 0.4).

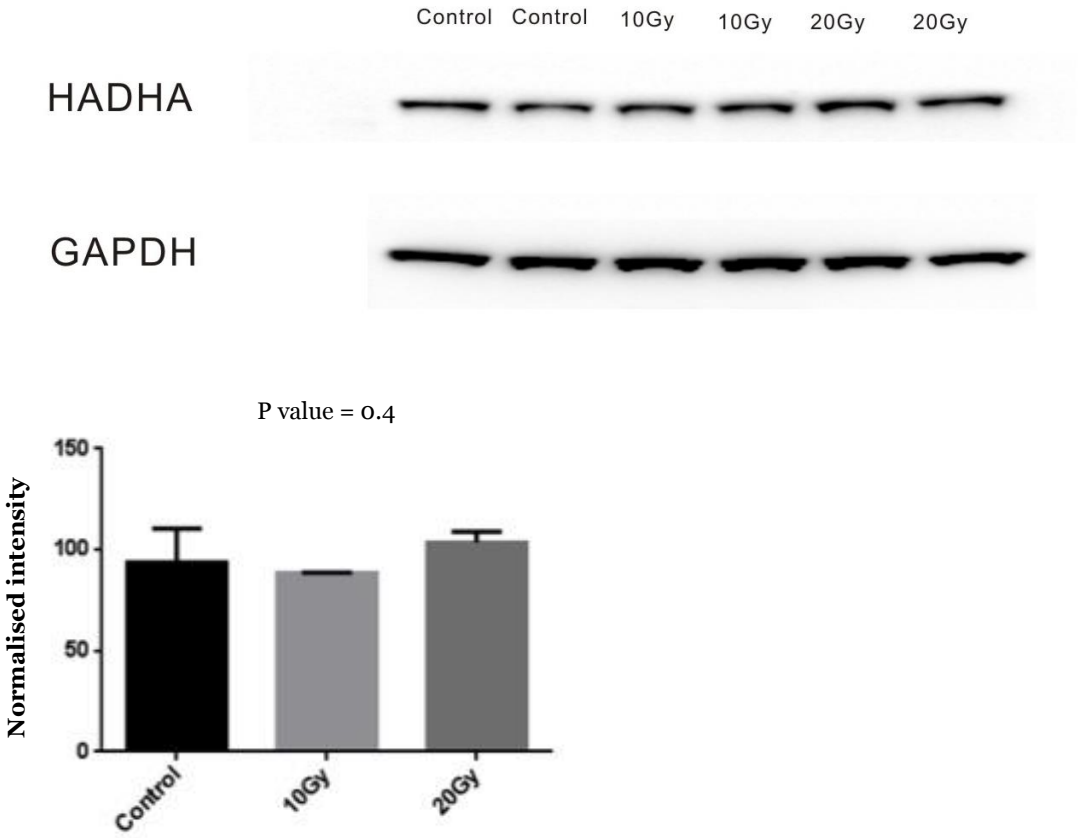

Figure 4.6: Western analysis of HADHA expression from HCMEC

At 48 hours post irradiation, the intensity of the HADHA bands were normalised against GAPDH band intensity for control, 10 Gy and 20 Gy samples. No significant difference (p>0.05) was observed between irradiated and control samples.

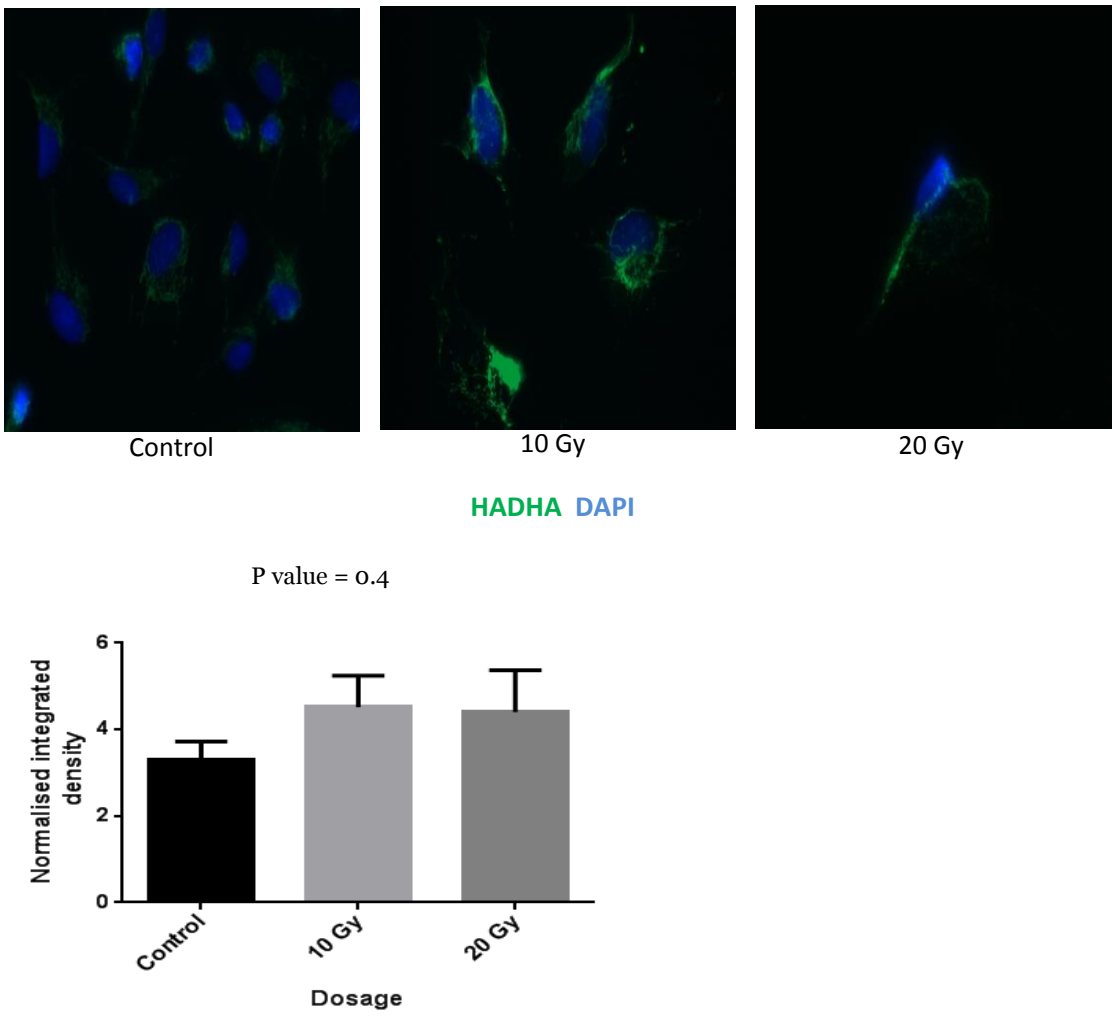

Figure 4.7: Immunofluorescence analysis of HADHA expression from HCMEC

At 48 hours post irradiation, cells were fixed with paraformaldehyde and the fluorescence intensity (normalised integrated density) due to HADHA was determined. Donkey anti-rabbit secondary antibodies were attached to Alexa Fluor 647 fluorophores. No significant differences ($p>0.05$) were observed between irradiated and control samples.

Elongation factor 2

Increased relative abundance of elongation factor 2 was observed in the radiated samples as compared to the control samples with a p-value of 0.0023 and fold change of 12 for the control vs. 10 Gy treatments (Table 4.1) and a p-value of 0.022 and fold change of 9 for the control vs. 20 Gy treatments (Table 4.2). Elongation factor 2 (EF2) was more abundant in both 10 Gy and 20 Gy treatments in the proteomic data when compared to control. No differences were observed, however in both western blot (p = 0.2) and immunofluorescence (p = 0.19) analyses (Fig. 4.8 and 4.9).

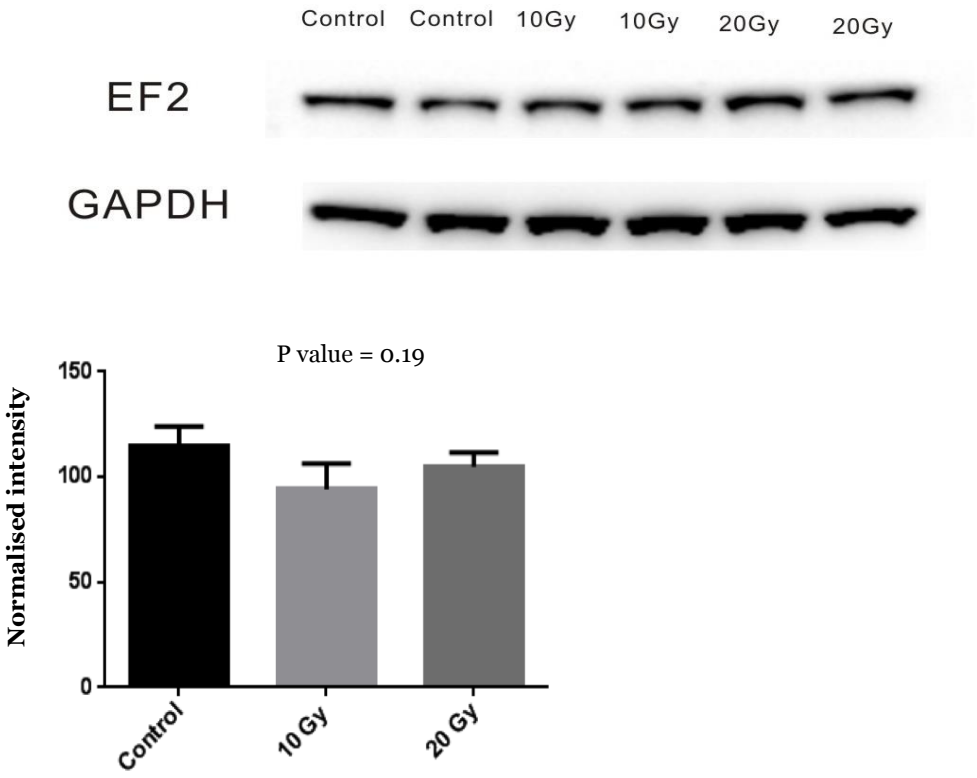

Figure 4.8: Western blot analysis of EF2 expression from HCMEC

At 48 hours post irradiation, the intensity of the EF2 bands were normalised against GAPDH band intensity for control, 10 Gy and 20 Gy samples. No significant difference (p>0.05) was observed between irradiated and control samples.

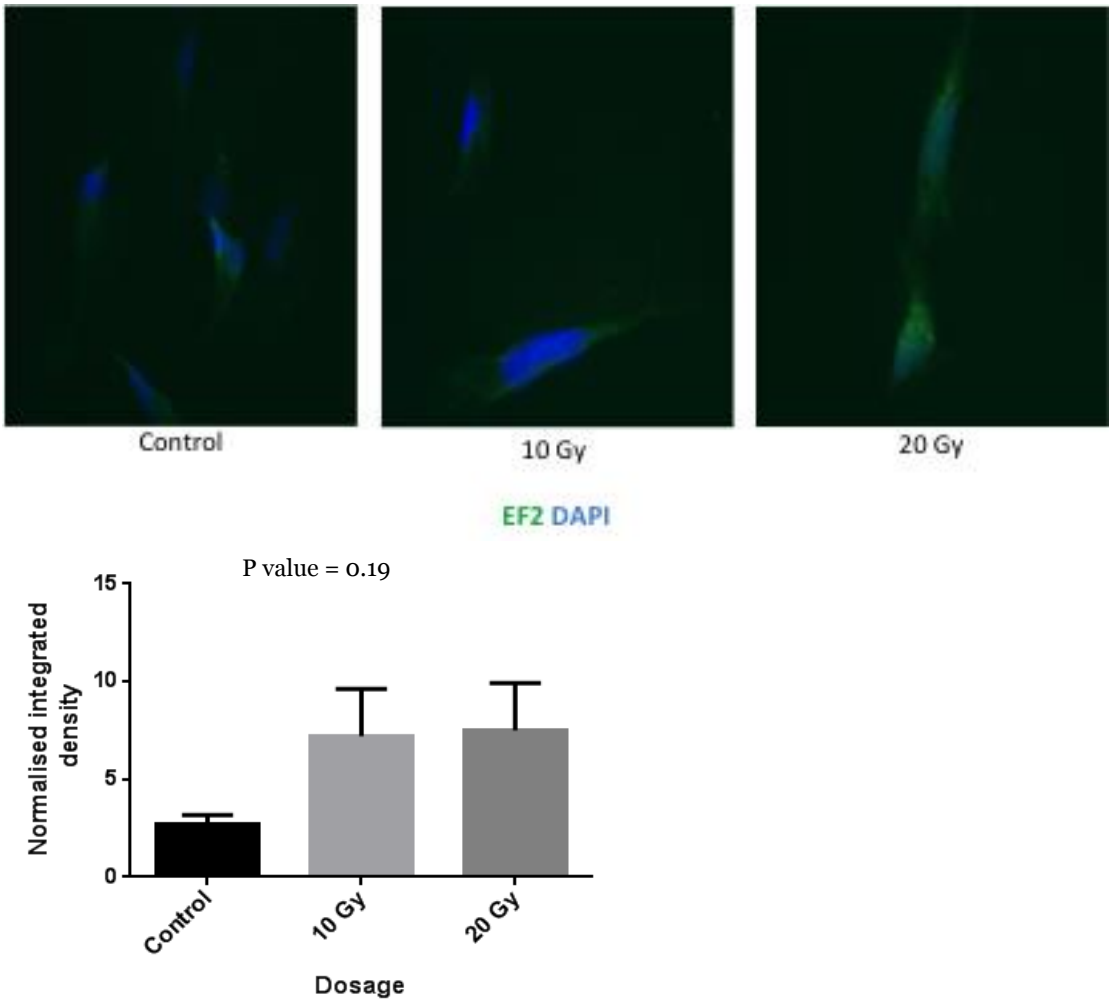

Figure 4.9: Immunofluorescence analysis of EF2 expression of HCMEC
At 48 hours post irradiation, cells were fixed with paraformaldehyde and the fluorescence intensity (normalised integrated density) due to HADHA was determined. Donkey anti-rabbit secondary antibodies were attached to Alexa Fluor 647 fluorophores. No significant differences ($p>0.05$) were observed between irradiated and control samples.

A summary of the results is presented below as Table 4.3.

Table 4.3: Summary of Results

PROTEIN	LC-MS-MS		IMMUNO		WESTERN BLOT	
	10 Gy	**20 Gy**	**10 Gy**	**20 Gy**	**10 Gy**	**20 Gy**
Talin	↑[a]	↑[a]	↑[b]	_[b]	_[c]	↑[c]
Myoferlin	_[a]	_[a]	↑[b]	↑[b]	_[c]	_[c]
HADHA	↑[a]	_[a]	_[b]	_[b]	_[c]	_[c]
EF2	↑[a]	↑[a]	_[b]	_[b]	_[c]	_[c]

↑- up regulated
_ - no significant change
a - Based on NSAF values, radiated samples relative to control
b - Based on integrated intensity, radiated samples relative to control
c - Based on normalised band intensities, radiated samples relative to control

Discussion

In order to increase the sensitivity of target detection in LC/MS/MS assays, cells were labelled with the synthetic biotin N-hydroxysuccinimide ester [23-26]. The subsequent capture of labelled proteins increased the likelihood of identifying low abundance proteins in biological samples. In addition to membrane proteins, the LC/MS/MS assays identified several cytoplasmic and a few mitochondrial proteins. Although our labelling method targeted cell surface proteins, the identification of intracellular proteins in our samples suggests that radiation-induced cell lysis has caused these proteins to be bound to the cell surface and be detected along with membrane proteins.

LC/MS/MS assays were used to identify possible vascular targets for SRS. A number of the proteins in our data set were further evaluated using western blot and immunofluorescence microscopy analyses.

Talin

Radiation treatment of HCMEC showed increased relative abundance of talin in both 10 Gy and 20 Gy when compared to control in the LC/MS/MS analysis. Increased intensity was observed in both western and immunofluorescence analyses.

Talin is a cytoskeletal protein, which is ubiquitous in distribution. This 270-kDa protein has talin-1 and talin-2 isoforms [27, 28]. The former, which is expressed in hematopoietic and endothelial cells, was investigated in this study. Talin is reported to be overexpressed in metastatic cells and tissues [29-33]. Its over expression has been reported in hepatocellular carcinoma, glioblastoma multiforme, ovarian serous carcinoma and ovarian squamous carcinoma [31, 34, 35]. Bostanci *et al* reported aberrant levels of talin in blood serum samples from patients with colon cancer, suggesting its potential to be an effective screening tool for the early detection of colon cancer [36]. Due to its relevance in cancer related pathways, talin is being investigated for the treatment of cancer.

Data from other studies showed that the relative abundance of talin is decreased after radiation treatment. Change in the relative abundance of the talin protein in

1, 2 and 4 Gy-irradiated human lymphocytes was identified by 2D-PAGE and MS analyses [37]. In a tissue specific study, which investigated radiation induced lung injury during thoracic cancer treatments, rats were irradiated at a dose of 28 Gy and the tissues were investigated 6 – 8 weeks post-irradiation. In both of these studies, talin was found to be down-regulated [38].

To the best of our knowledge, the changes in the relative abundance of talin in irradiated human brain endothelial cells have not been reported previously. Talin's role in the activation of integrin transmembrane proteins is supported in our study, where both integrin α-5 and integrin β -1 have been detected in both 10 Gy and 20 Gy samples [39]. However both integrin α-5 and integrin β-1 are less abundant when compared to control samples in the LC/MS/MS analyses. Integrin α-3 shows more abundance in 20 Gy treatments and integrin β-3 shows more abundance in both 10 Gy and 20 Gy treatments. Talin plays a vital role in cell adhesion and cell migration. It binds to integrin protein complexes and activates them [39]. The immunofluorescence data do not show talin getting externalised to the outer surface of the cell. It would be interesting however to see the localisation and expression levels of integrin β in radiated human brain microvascular endothelial cells.

Myoferlin

Myoferlin is a 230-kDa membrane protein belonging to the ferlin family of proteins [40]. Ferlin proteins have long been associated with muscle tissues since they were first reported in muscle fibres [41-43]. These muscle specific proteins are expressed in high levels in vascular endothelial cells carrying out the basic functions of endothelial cells such as cell proliferation and homeostasis [40].

Myoferlin has been reported to be at varied abundance levels in human and mouse breast cancer cell lines when compared to control COS-7 cells. Increased expression levels have been observed in solid mouse and human carcinoma tissues by western analyses [40]. Increased expression levels have also been reported in tumour tissues resected from patients with oropharyngeal squamous cell carcinoma [44].

To the best of our knowledge, no studies relating to irradiation have been carried out in myoferlin. Increased levels of myoferlin in muscle fibres are expressed as means of cell repair in response to stress and remodelling [40]. We would expect increased levels of myoferlin in irradiated cells due to the stress caused by radiation. But our western blot results show no difference in the pattern of expression between the sample groups (Fig. 4.4).

Myoferlin, which is a membrane protein, was not externalised post radiation (Fig. 4.5). The localisation rather is very perinuclear and cytoplasmic as observed by Huang *et al* in their study on vascular endothelial cells and by Leung *et al* in their study on cultured mouse Lewis lung carcinoma cells [40, 45].

The correlation of myoferlin expression to vascular endothelial growth factor (VEGF) has been reported in cancer studies where increased myoferlin expression levels had an effect on increased secretion of VEGF [46, 47]. In a study by Bernatchez *et al*, myoferlin was highly expressed in surface biotinylated endothelial cells. Silencing of myoferlin resulted in lack of endothelial cell proliferation in response to VEGF [47]. Elevated levels of VEGF have been reported in the AVM endothelium [48-50]. Whether myoferlin plays a role in elevated levels of VEGF in the AVM endothelium cannot be determined here as this study has a limitation of being conducted in cell lines and not from AVM primary cultures.

Although this study does not give conclusive findings on myoferlin, it may be good to validate the molecule in animal models. Fahmy *et al*, in their study on pancreatic ductal adenocarcinoma tissues, reported the density of blood vessels to be increased in myoferlin positive tissues and reduced in myoferlin negative tissues [46]. Investigating the blood vessels from animal models can shed light on the expression of myoferlin in the AVM endothelium. In addition to primary phenotypical variances, the AVM endothelium varies in the molecular level from normal endothelium due to the stress caused by the high-pressure blood flow through the cerebral veins. Animal models of AVMs that mimic AVM

haemodynamics could be investigated for the expression of myoferlin before eliminating this target molecule from the potential list of vascular targets.

HADHA

Radiation treatment of HCMEC showed increased relative abundance of HADHA in 10 Gy treatments when compared to control. No significant differences were observed in intensity levels in both western and immunofluorescence analyses.

HADHA, a mitochondrial 83-kDa protein is a subunit of the mitochondrial trifunctional protein family [51]. *In vitro* and *in vivo* studies have been conducted on HADHA in the field of cancer treatment. Up-regulated expressions of the protein have been reported in lung cancer, non-metastatic breast cancer and metastatic breast cancer and down regulated expressions in hepatocellular carcinoma and clear cell renal carcinoma [51, 52].

There has been no study on the expression levels of HADHA post-irradiation. Radiation causes cells to go through senescence or death. DNA damage however is the primary effect it has on cells, which may lead to mutations and these mutations could be mitochondrial specific [51]. This could be the reason for HADHA and other mitochondrial proteins to be detected in our LC/MS/MS analysis. HADHA however was up-regulated only in the 10 Gy samples, which could suggest that HADHA expression is dose dependent. But our western blot and immunofluorescence analyses showed no significant differences between the radiated and control samples (Fig. 4.6 and Fig. 4.7). In the immunofluorescence data, other than senescence, which is a phenotypic alteration caused by radiation in cells, no translocation of the protein was observed in the radiated samples.

Elongation factor 2

Radiation treatment of HCMEC showed increased relative abundance of elongation factor 2 (EF2) in both 10 Gy and 20 Gy treatments when compared to control. No significant differences were observed in intensity levels in both western and immunofluorescence analyses.

EF2 plays an essential role in protein synthesis and is being investigated for possible use in cancer therapeutics [53]. In a proteomic study on aged rat astrocytes, EF2 was up-regulated 3 hours after irradiation at a dosage of 0.1 Gy and dropped down at 24 hours post-irradiation. This study, which investigated the correlation of radiation treatment with ageing, is the only research reported so far on the expression of EF2 post-irradiation [54]. In our data acquired from LC/MS/MS analysis, EF2 was significantly up-regulated in both the radiated groups. Among the list of significant proteins, the maximum fold changes (12 for 10 Gy and 9 for 20 Gy) were observed in EF2 protein when compared against controls (Table 4.1 and Table 4.2). For this reason, we selected this molecule for further validation by western blot and immunofluorescence analyses. There was no difference between the radiated and control groups in both validating analyses (Fig. 4.8 and Fig. 4.9).

Limitations of the study

The cell extract samples analysed by LC/MS/MS were biotin labelled which enhanced the capture of low abundance proteins. The cell extract samples used for the western blot and immunofluorescence analyses however were from whole cell lysates, which may be the reason the expression patterns in western blots are not consistent with the LC/MS/MS results for myoferlin, HADHA and EF2. Future related work could be done on biotin labelled cells to keep the same conditions for both proteomic and validation analyses. The other limitation of this study is that it investigates the radiation-induced changes in human cerebral microvascular endothelial cells from a cell line. A similar study carried out in AVM primary culture would give more clarity.

In conclusion, our discovery of the increased relative abundance of talin is promising for its possible role in vascular targeting. Although increased fluorescent intensity levels have been observed in the immunofluorescence analysis, the sub-cellular location of talin post-irradiation is unclear because the cells used in this assay were permeabilised. Once this is confirmed, further work on the prospective use of talin for vascular targeting could be carried out.

References

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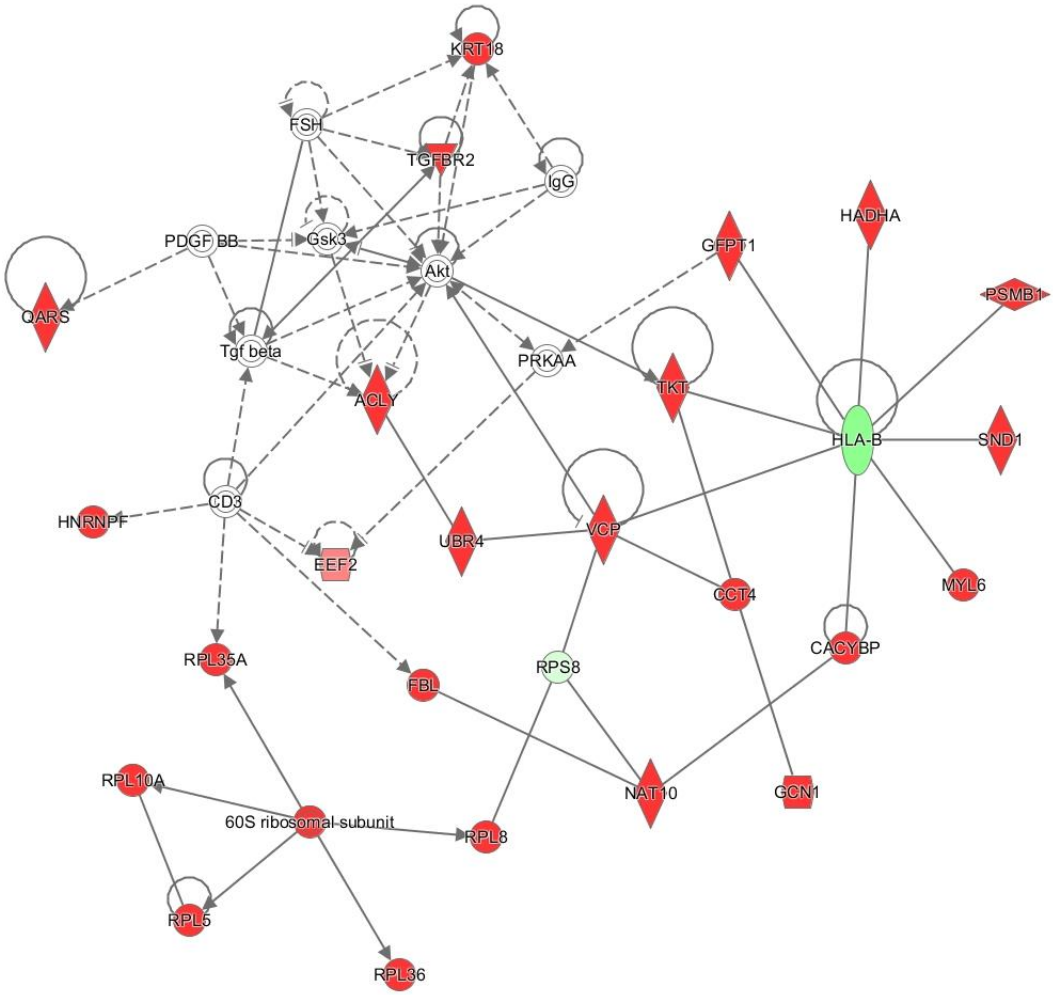
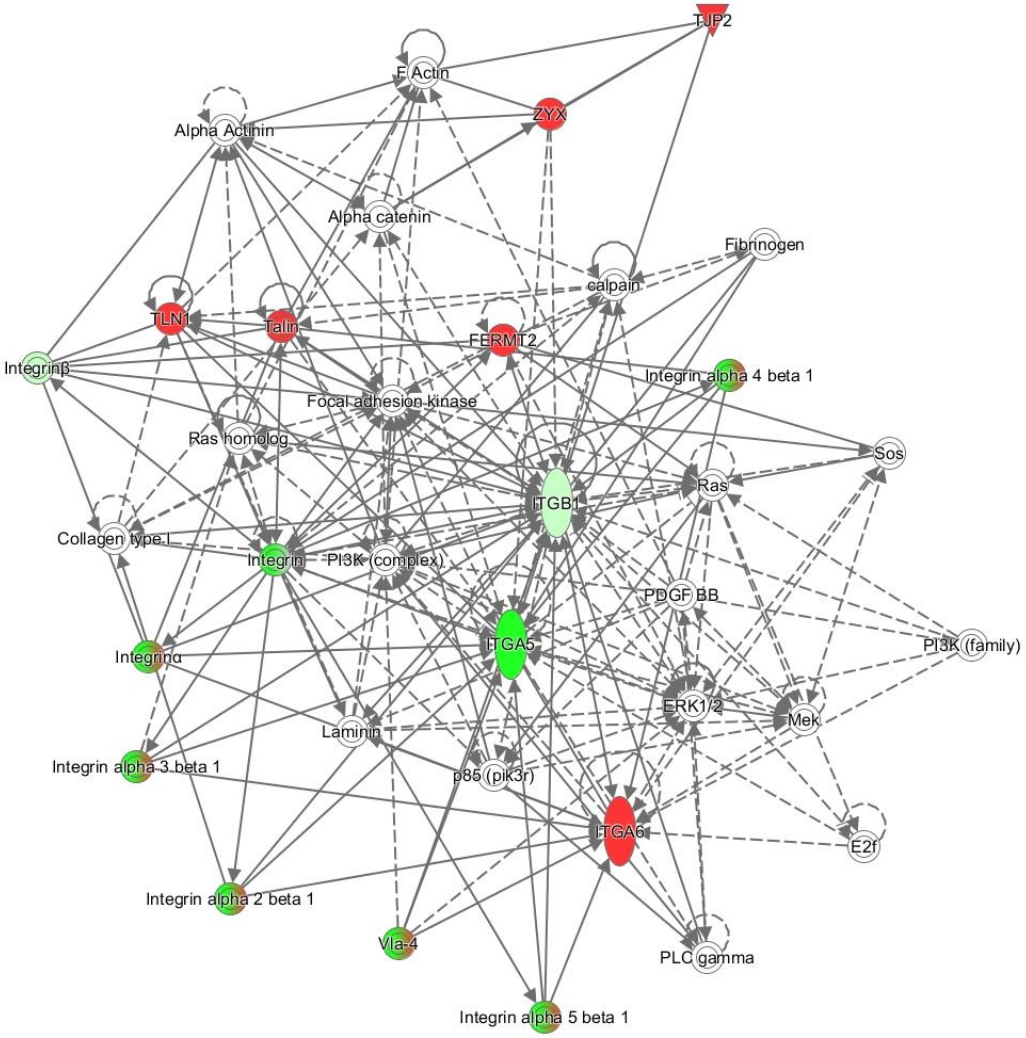
Supplementary Figures and Tables



Supplementary Figure 4.1: Network 1 – 10 Gy vs. Control of HCMEC at 48 hours post irradiation

HADHA, which was increased in its relative abundance in the non-spectral abundance factor analysis, is shown to be increased in its relative abundance in this pathway when compared between the 10 Gy and control samples. The red colour represents proteins that are increased in their relative abundance. Interestingly, the expression of HADHA did not differ between irradiated and control samples in both western blot and immunofluorescence analyses.



Supplementary Figure 4.2: Network 3 – 20 Gy vs. Control of HCMEC at 48 hours post irradiation

Talin that was increased in both 10 Gy and 20 Gy samples in the non-spectral abundance factor analysis is shown in this pathway. Talin shows a link with different integrin proteins that are decreased in abundance. The red nodes represent proteins that are increased in relative abundance and the green nodes represent proteins that are decreased in relative abundance. Talin showed significant difference between the irradiated and control samples in both western blot and immunofluorescence analyses. Fermitin, which is linked to talin in this pathway, can be a potential target for evaluation in further analysis.

Supplementary Table 4.1: Ingenuity Pathway Analysis of Associated Network Functions for 10 Gy Treatments

ID	Associated Network Functions	Score
1	Cancer, cell death and survival, organismal injury and abnormalities	61
2	Cellular assembly organisation, cellular function and maintenance, cell-to-cell signalling interaction	46
3	DNA replication, recombination and repair, cellular assembly and organisation, cellular compromise	35
4	Auditory disease, hereditary disorder, neurological disease	13
5	Carbohydrate metabolism, lipid metabolism, small molecule biochemistry	11

Supplementary Table 4.2: Ingenuity Pathway Analysis of Associated Network Functions for 20 Gy Treatments

ID	Associated Network Functions	Score
1	Cell cycle, cell morphology, lipid metabolism	39
2	Cancer, organisational injury and abnormalities, amino acid metabolism	22
3	Tissue development, cell morphology, digestive system development and function	14

Chapter 5

Conclusion

Brain AVMs cause intracerebral haemorrhage in children and young adults often leading to death or disability [1-3]. Currently, brain AVMs are treated by surgery, radiosurgery or embolisation [4-7]. A subset of AVMs that are large and located in critical regions of the brain cannot be treated with existing options [8-10]. There is therefore a need for a novel treatment for those AVMs that remain a challenge for treatment with existing therapies.

A novel and molecular based treatment called vascular targeting is proposed for treating AVMs. In this technique, a protein molecule on the AVM vasculature will be targeted with an antibody. The antibody will be attached to an effector molecule such as thrombin or tissue factor to promote thrombosis. This technique has been used in the treatment of cancer and has proved effective [11]. The endothelium of tumour vasculature is very different to the normal endothelium, which helps in targeting the tumour vasculature in the treatment of cancer [12]. This unfortunately is not the case in AVMs; AVM vasculature does not differ significantly from the normal endothelium [13-17]. In order to overcome this limitation, radiosurgery is proposed to 'prime' the endothelial cells of the AVM vasculature [15, 16, 18]. Radiosurgery has the advantage of targeting a particular volume with minimal effect on the surrounding tissues [19]. The overall aim therefore is to employ radiation to induce molecular changes in the AVM endothelium and discover a target molecule that could be used for vascular targeting.

For vascular targeting to work in AVMs, a discriminating target molecule must be identified. It is not feasible to carry out the process of identifying such a target in humans. Therefore *in vitro* and *in vivo* work is being done. At present there is no single experimental model that can provide all answers pertaining to the identification of specific target molecules, therefore identification of a suitable target will require the use of multiple techniques. The identified target molecule should have the following characteristics to be used in the treatment of AVMs. Firstly, it should be localised to the radiated tissue. Secondly, it should be expressed on the cell surface and thirdly, it should be sufficiently discriminating from normal vessels, i.e. it should significantly be increased in abundance in the

irradiated vessels. The work described in this thesis was performed in pursuit of suitable targets.

Summary of results

The response of endothelial cells to irradiation was studied both *in vitro* and *in vivo* through proteomic-based techniques in earlier work [20]. Three targets – CD51, CD109 and BMP3 were selected from the list of protein targets increased in relative abundance after irradiation for further analysis in this thesis. Immunohistochemical analysis was carried out in AVM vessels and common carotid artery (CCA) tissues extracted from animal models to investigate the location of CD51, CD109 and BMP3 proteins in the endothelial cells. The analysis revealed the presence of CD51 in AVM vessels from days 1 and 3 after irradiation and was absent in AVM vessels from days 7, 21 and 42 after irradiation. CD51 was absent in the non-irradiated AVM vessels. However, CD51 was present in both control and irradiated tissues of the CCA. CD109 was not detected in the endothelium in any of the experimental groups. BMP3 was present in the endothelium on day 21 after irradiation of both AVM vessels and CCA tissues. BMP3 was not present in any of the control tissues. On the basis of these findings, BMP3 looks to be a very promising molecule for further investigation. However, it was not apparent if the data obtained in the study of the animal model (i.e. the relative abundance of CD51, CD109 and BMP3) might be extrapolated to humans. While the animal model replicates the haemodynamics of human AVM, it has several limitations [21]. As the immunohistochemical analyses were carried out in fixed tissues, it was not possible to obtain precise locations of these proteins on the surface of the endothelial cells in the sections.

In order to further explore the endothelial molecular changes in AVMs, *in vitro* and *in vivo* studies were followed by investigating humans with AVMs. It is not feasible to extract AVMs from humans early after radiosurgery. There is the potential to get an insight into the endothelial molecular changes in the blood. Changes may be detected in the endothelial microparticles (EMPs). EMPs have been reported to be elevated in various diseased states [22]. There has been no report in literature on the correlation of EMPs and AVMs. EMPs from plasma samples were analysed using flow cytometry and compared to samples from

healthy volunteers. The protein, CD51 was observed to be elevated in the plasma of AVM patients. The other protein, CD105 which is elevated in HHT gene mutations, a genetic condition associated with AVMs was observed to be decreased in AVM patient plasma samples when compared to healthy volunteer samples. These changes in the relative abundance of the proteins in the EMPs were not statistically significant. The sample size was a major limitation in this study. (Additional samples will be analysed using flow cytometry in ongoing work. It is hoped that the larger sample size will increase the statistical robustness of the findings.)

To date, the work on AVMs conducted in this laboratory has used cultured mouse brain endothelial cells, as well as animal models of AVM. To better understand the response of human brain endothelial cells to irradiation, the final aim of this project was to determine radiation-induced protein expression in human brain microvascular endothelial cells. Two potential targets, CD109 and BMP3 that were detected in earlier proteomic studies of mouse brain endothelial cells were not detected in the proteomic analysis of human brain endothelial cells [20]. Integrin proteins were the only membrane proteins in the list of significantly up-regulated proteins in the irradiated samples. Three proteins from this list – Talin, HADHA and elongation factor 2 and myoferlin, a membrane protein (not statistically significant) were selected for further validation by western blot and immunofluorescence analyses. Talin was the only protein that showed increased band intensity in the radiated samples in western blot analysis. Likewise, increased fluorescence intensity was observed in the irradiated cells during immunofluorescence analysis. However, as the cells were permeabilised during sample preparation for immunofluorescence microscopy, the sub-cellular location of the protein could not be precisely determined. All other three proteins (myoferlin, HADHA and EF2) showed no significant difference between irradiated and control samples. The reason for the difference in pattern between the western blot and the proteomic techniques could be attributed to differences in sample preparation. Samples were biotinylated to enrich the capturing of membrane proteins for LC/MS/MS analysis whereas whole cell lysates were used for western blot and immunofluorescence analyses.

Ideally, it would have added more value if the same molecules were validated in the animal model for expression. This will be evaluated in future studies. This study only gives the expression of proteins in human cerebral microvascular endothelial cells in response to irradiation. This study was carried out on cell line culture and not primary AVM endothelial culture. This is a major limitation of the study. Developing primary cultures of human AVM endothelium is part of ongoing studies.

In summary, out of the seven target molecules, the protein BMP3 shows localisation in irradiated tissues of day 21 in the animal model. The irradiated cells show increased expression of talin in LC/MS/MS, western blot and immunofluorescence analyses.

Future directions

Further work on circulating EMPs by investigating their proteome would add to the existing knowledge on the molecular biology of AVMs. This could be done in plasma samples from both AVM patients and the animal model. Plasma samples could be collected at different time points post-irradiation. If a similar proteome is observed in plasma samples of both AVM patients and non-irradiated AVM animal models, it can be expected to observe similar patterns in human AVMs after irradiation as what is observed in the animal models exposed to irradiation.

The use of plasma for studying biological functions and malfunctions is mainly because of the insight it gives of the human proteome [23]. High abundance proteins, which are over 99% of the total proteins, mask the detection of low abundance proteins in most proteomic analyses [24, 25]. This was a problem in the current work, where the high abundance proteins such as actin, lipopolysaccharide binding protein and heparin cofactor 2, masked all other proteins that may have been of interest for further analysis. It is the low abundance proteins that give further perception of apoptosis, inflammation, cell damage, cell signalling and tissue leakage [26, 27]. Enhancing the detection of low abundance proteins could be done by immunodepletion. Over twenty high abundance proteins have been able to be depleted by commercially available columns [26, 27]. If EMP count in AVM patient plasma samples is significantly

elevated to control samples in future work, immunodepletion could be sought to better detect those proteins that could not be detected in this study. In this way, a better understanding of the proteomic signature in AVM patients could be perceived.

Overall, the work described in this thesis points to talin and BMP3 as being promising candidates for vascular targeting. However, further validation of the molecules is needed. This could be done in more human and non-human studies. If the molecules prove to be successful in producing intravascular thrombosis in the animal model, it will be required then to demonstrate that radiosurgery in humans also creates a similar molecular signature. This could be verified using PET scan and microparticle-related proteomic studies. It will also be required to create a larger animal model to be sure of the safety of the technique of producing thrombosis in the brain, which will then finally be followed by clinical trials.

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Publications and presentations

Publications arising from this thesis

Amal Raj J, Stoodley M. Experimental animal models of arteriovenous malformations: A Review. *Veterinary Sciences* **2015**, 2(2), 97-110.

Amal Raj J, Combes V, Nair S, Stoodley M, Davidson A. Circulating endothelial microparticles in patients with cerebral arteriovenous malformation (In preparation).

Amal Raj J, McRobb L, Lee V, Wiedman M, Grace M, Moutrie V, Stoodley M. Expression of CD51, CD109 and BMP3 in an animal model of arteriovenous malformation after radiosurgery (In preparation).

Amal Raj J, McRobb L, Wiedman M, Moutrie V, McKay M, Stoodley M. Identification of radiation-induced membrane proteins in human cerebral microvascular endothelial cells for the treatment of brain arteriovenous malformations (In preparation).

Publications related to work not presented in this thesis

Kashba S, Patel N, Grace M, Lee V, Raoufi-Rad N, **Amal Raj J**. Duong H, Stoodley M. Angiographic, hemodynamic and histological changes in an animal model of brain arteriovenous malformations treated with Gamma Knife radiosurgery. *Neurosurgery* **2015** Published online April 17, 2015

McRobb L, Lee V, Simonian M, Zhao Z, Thomas S, Wiedmann M, **Amal Raj J**, Grace M, Moutrie V, Mackay M, Molloy M, Stoodley M. Radiosurgery alters the endothelial surface proteome: Externalized Intracellular molecules as potential vascular targets in irradiated brain arteriovenous malformations. Accepted at *Journal of Radiation Research*.

Raoufi-Rad N, McRobb L, Zhao Z, Lee V, Patel N, Qureshi A, Grace M, McHattan J, **Amal Raj J**, Duong H, Kashba S, Stoodley M. Phosphatidylserine translocation following radiosurgery in an animal model of arteriovenous malformation. Under revision at *Journal of Radiation Research.*

Conference Presentations

Amal Raj J, McRobb L, Lee V, Wiedmann M, Grace M, Moutrie V, Stoodley M. Molecular changes in arteriovenous malformations treated with radiosurgery. Digital Poster presented at the 'AANS/CNS Joint Cerebrovascular Section Annual Meeting', Los Angeles, United States of America, February, 2016.

McRobb L, Lee V, Thomas S, Wiedmann M, **Amal Raj J**, Zhao Z, Grace M, Moutrie V, Mckay M, Molloy M, Stoodley M. Radiosurgery induces novel protein changes on the endothelium of arteriovenous malformations: potential for therapeutic targets. Poster presented at the 'Stroke' Conference, Los Angeles, United States of America, February, 2016.

Amal Raj J, Nair S, Combes V, Stoodley M, Davidson A**.** Evaluation of circulating endothelial microparticles in patients with arteriovenous malformations. Poster presented at the 'Australasian Neuroscience Society' Conference, Adelaide, Australia, January 2014.

Kashba S, Patel N, Duong N, Grace M, Lee V, Raoufi-Rad N, **Amal Raj J**, Stoodley M. Gamma Knife induced hemodynamics and angiographic in animal model of AVM. Poster presented at AANS Annual Scientific Meeting, New Orleans, Louisiana, United States of America, April-May, 2013.

Amal Raj J, Davidson A, Molloy M, Nair S, Stoodley M. Understanding the molecular changes in human brain arteriovenous malformations using circulating endothelial microparticles. Poster presented at the 'Proteomics and beyond' Symposium, Sydney, Australia, November 2012.

Appendix

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/051 -3 **Date of Expiry:** 31 December 2015

Full Approval Duration: *01 January 2015 to 31 December 2015*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Associate Investigator:

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Marcus Stoodley	0407 896 492
Vivienne Lee	0416 250 779
Markus Wiedmann	0431 976 760

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Manager, CAF: 9850 7780 / 0428 861 163 and **Animal Welfare Officer**: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Immunohistochemical analysis of radiation-induced protein changes in a rat model of arteriovenous malformation (AVM).**

Purpose: **4 - Research: Human or Animal Biology**

Aims: **To develop new treatments for brain arteriovenous malformations (AVMs) that are safer and more effective than current methods of surgery and radiosurgery.**

Surgical Procedures category: 5 - Major Surgery with Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex/Age/Weight	Total	Supplier/Source
02 Rattus	Sprague Dawley	Male/6 weeks on arrival	40 +6	ARC Perth
		TOTAL	46	

Location of research:

Location	*Full street address*
ASAM	Level 1, F10A, 2 Technology Place, Macquarie University, NSW 2109
CAF	Building F9A, Research Park Drive, Macquarie University, NSW 2109

Amendments approved by the AEC since initial approval:
1. Amendment #1: Addition of Markus Wiedmann as Associate Investigator (Exec approved, ratified by AEC 19 March 2015)
2. Amendment #2: Request addition animals (Exec approved, ratified by AEC 14 May 2015)

Conditions of Approval: N/A
1, Amendment #1: Markus Wiedmann to complete and passing the Research Animal Care and Ethics (RACE) Training 2015.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date: 14 May 2015**

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2012/029 - 5

Date of Expiry: 20 May 2015

Full Approval Duration: 21 May 2012 to 20 May 2015 (36 months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry.**

Principal Investigator:
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Associate Investigators:

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Marcus Stoodley	0407 896 492
Andrew Davidson	0416 157 859
Vivienne Lee	0416 250 779
Newsha Raoufi-rad	0415 599 087
Saleh Kashba	0415 789 600

In case of emergency, please contact:
Animal Welfare Officer 9850 7758 / 0439 497 383
Manager, CAF 9850 7780 / 0428 861 163
or the Principal Investigator / Associate Investigator named above

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: To investigate the endothelial molecular changes by the detection of endothelial mircroparticles (EMPS) in AVMS treated with and without radiosurgery

Purpose: 4 - Research (human or animal biology)

Aim: To investigate the endothelial molecular changes by the detection of endothelial mircroparticles (EMPS) in the plasma of rats with AVMS treated with and without radiosurgery

Surgical Procedures category: 5 (Major Surgery With Recovery)

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex	Weight	Age	Total	Supplier/Source
Rattus norvegicus	Sprague-Dawley	male	250-500g	6-12 weeks	6	ARC Perth
Rattus norvegicus	Sprague-Dawley	male	250-500g	6-12 weeks	28	ARC Perth
				TOTAL	34	ARC Perth

Location of research:

Location	***Full street address***
Australian School of Advanced Medicine	Level 1, Clinic Building, 2 Technology Place, Macquarie University NSW 2109
Central Animal House Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109
Macquarie University Hospital	3 Technology Place, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval:

1. Omitting blood collection by cardiac puncture, change in less animal number (Exec approved 18 March 2014, ratified by AEC 20 March 2014)
2. Removal of Dr Hong Duong and addition of Dr Zhenjun Zhao as Principal Investigator (Exec approved, ratified by AEC 18 September 2014)

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee)

Approval Date: 18 September 2014

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MACQUARIE University
SYDNEY·AUSTRALIA

30 May 2011

Dear Dr Davidson

Reference No: 5201100266

Title: Understanding the molecular changes in human brain arteriovenous malformations using circulating endothelial microparticles

Thank you for submitting the above application for ethical and scientific review. Your application was considered by the Macquarie University Human Research Ethics Committee (HREC (Medical Sciences)).

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated May 2015) (the *National Statement*).

Standard Conditions of Approval:

1. Continuing compliance with the requirements of the *National Statement,* which is available at the following website:

http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. All adverse events, including events which might affect the continued ethical and scientific acceptability of the project, must be reported to the HREC within 72 hours.

4. Proposed changes to the protocol and associated documents must be submitted to the Committee for approval before implementation.

It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email ethics.secretariat@mq.edu.au

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely

Professor Tony Eyers
Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.