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School of Life, Health and Chemical Sciences

Age-related alterations on ultrastructure and gene expression profile of the female blood-brain barrier

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**A Thesis submission to the Open University for the degree of
Doctor of Philosophy**

February 2020



Declaration

I declare that the work presented in this thesis is my own and contributions made by other researchers are acknowledged in relevant parts of the text. This work does not contain any material submitted for any award or other degree.

Abstract

Blood-Brain Barrier (BBB) breakdown occurs in ageing and neurodegenerative disorders and affects several brain regions including cortex and hippocampus. During ageing, structural and functional changes affecting the main BBB components (brain endothelial cells (BECs), pericytes and astrocytes), appear to be associated with altered expression of genes and microRNAs (miRNAs) potentially related to development, protein synthesis or longevity pathways. However, little is known about the age-related BBB dysfunction in females. In this study, we aimed to assess the relation between ultrastructural and transcriptional changes in the ageing female BBB. A combination of transmission electron microscopy (TEM) and 3D reconstruction was used to study microvessel ultrastructure in 6- and 24-month-old female C57BL/6J mice. According to our results, the ageing female BBB shows a significant increase in basement membrane (BM) thickness, volume and number of BEC pseudopods, pericyte mitochondrial volume, pericyte – BEC contact and tight junction (TJ) tortuosity. Also, cortical capillaries appeared to be prominently more affected during ageing than hippocampal capillaries. These results suggest a higher impact of ageing on the cortical BBB in females, promoting changes that lead to a pro-inflammatory state, among other processes. In addition, sequencing results showed that the majority of upregulated genes in the ageing female BBB were involved in inflammation and immune response pathways, whereas the downregulated genes were mostly related to metabolism and signalling pathways. Amongst the age-deregulated mRNAs and miRNAs, miR-144-3p (upregulated) and *Dnmt3a* (downregulated) were selected for functional analysis in a human BEC line (hCMEC/D3), where their inverse correlation was confirmed. However, *DNMT3A*, not miR-144-3p, was shown to influence BEC function when deregulated, thereby promoting higher leukocyte adhesion and mRNA levels of adhesion molecules (*ICAM-1*, *VCAM-1*) and the chemokine *CCL5*. Age-induced increase of miR-144-3p appears to modulate *DNMT3A* expression, but *DNMT3A* might be independently contributing more to switching BECs into a pro-inflammatory state.

Acknowledgments and dedications

This thesis is the result of three and a half years of challenging work and would not have been possible without the help and support of the following people. I would like to especially thank my supervisors Nacho Romero, Cheryl Hawkes and Francesco Crea. Nacho, first of all, thank you for giving me this opportunity and guiding me on my first steps in academia and the real world, thank you as well for all the help and advice throughout my PhD journey, you really are one of a kind; you have taught me to think out of the box and to push my boundaries to become a better researcher, and for that I will always thank you. Cheryl, I will be forever grateful for having you as my second supervisor, you are a true hero and every scientist should aspire to be as smart, disciplined and hardworking as you are; I will miss our long talks and your funny responses to my stressful days, I wish you all the best in your new position, I am sure you will nail it. Francesco, your support along my PhD has been greatly valuable, and I will always be grateful for what you have taught me. Although he is not one of my official supervisors, I would like to include David Male in this section. David, I have never met someone who works and dedicates themselves to science with such love and respect as you do, you are a real inspiration to us all; I will always remember all your good comments, your support and your help along my PhD training.

I would also like to take a moment to thank the BRU staff at the OU, Karen, Agata and Iwona, for helping me with everything related to the animal work and being as amazing and supportive as they are. Also, I would like to thank the EM staff, Igor and Radka, for their invaluable help with the nightmare that is the 3D reconstruction and for always making my life much easier. Of course, I would like to extend my thanks to the OU lab support staff, Julia, Brett, George and Eduardo for all their kindness, support and knowledge, as well as Jen, for being such a bright and great woman.

A PhD is not the same without other PhD students and colleagues to keep going with, thus I would also like to thank the rest of the BBB group including Jane, Joyce, David RC, Zerín, Mo, Conor, Perla, Rebecca and Nayab, for all the good seminars and stimulating, thought-provoking conversations. I would like to thank Ester, Sonia, Shereen and Laura in particular, for their support, their friendship and their unlimited patience, I will cherish all our adventures and moments together. Other students have been part of these years, especially Lewis, Morgane, Salah, Pelumi and Amy for being the best office

mates you can think of, and Tala, Emily B, Emily C, Stephen, Irene, Sarah, Gaurav and Sophie, and to Alexandra for the coffee machine that has helped me throughout the writing of this thesis. Also, as part of the OU, we are lucky to receive internship students, all of them incredibly great, thus I would also like to thank Marta, Sarai, Naiara, Maëva, Juan, Maurine, Marcelle and Léa, for bringing new energy and spirit into the lab during their time here.

I would also like to thank those friends out of the lab, but without whom the life in UK would have been much more boring. María, my housemate for almost 2 years and my friend for the rest of our lives, thanks for being my family and for your infinite support and kindness during my lowest moments. Miriam y Alba, thanks for those days of lunches and laughs, and for one of the craziest trips I have ever been to; I am sure our paths will cross again. Ellie, thanks for being a wonderful friend and even a better human being, I cannot wait to see you thrive as a professional scientist and be sure I will be there to celebrate with you. Daniel, first a colleague from another department, then a housemate and finally a friend, thanks for making that little house in Monkston a cosy home.

Collaboration is a key factor in science, therefore one of the best things of my PhD was being part of the European PhD Training Network BtRAIN. Thanks to everyone involved in this network, for three years filled with good training, new experiences and knowledge. I would like to especially thank Ricardo Figueiredo and Peter Winter from GenXPro for their help and support with the sequencing analyses. Also, I would like to thank Marie, Marjolein and Amaia, since their company and friendship have made this PhD journey way more magical. But also, and in addition to Ricardo, thanks to Sabela, David, Luca, Ana, Raoul, Nikos and Markus, the best people I could have shared this adventure with.

Let me take a moment to also thank the people that have supported me from the very beginning, including my group of crazy friends from Málaga: Clau, Elena, Esty, Migue, Leo, Estela, Wiki, Chema, Adriano, Ali and Fran. Thank you for believing in me even when I did not; I will always be grateful for having you in my life, and it does not matter how far away we are, I know we will always find our way to each other, I love you 3000. Also, thanks to Borja, Sara and Noe with whom I started in this world of neuroscience and research and who have supported me infinitely, I am very lucky getting to call you my friends. I would also like to thank Mari for all her lessons during my

master, and Mari Ángeles, my MSc supervisor, for giving me the first opportunity in a lab and for increasing my love for science.

And, of course, we are nothing without our family. Juan Antonio, it is not a secret that you are my favourite cousin, and having you living just right “next to me” in London made living far from home much easier, thanks for being there. I would like to extend my thanks to my cousin Javier and my aunties. Antonio, thanks for being a great husband to my sister and father to my nieces, together with our second family in Fuengirola, all your support helped me throughout the last few years. Mari Carmen and Esther, I cannot believe how fast you are growing up, it fills me with joy and pride seeing the little women you are becoming, I love you lots and I cannot wait to see the new awesome experiences that life will bring to you, you are a gift to us all, and the same goes for Laura and Oliver. Esther, my dear sister, you are unique, spectacular and amazing, and I cannot put into words how much I love you, be prepared to continue travelling the world together because you are my perfect travel partner. And, finally, the two people without whom I would have not gotten this far, Antonia and Eduardo, my parents, thank you both for all your love, kindness, life lessons, infinite support and unbreakable faith in me, all this work is undoubtedly dedicated to you, for I am who I am today because of you. I love you to the moon and back (*os amo infinito*).

Finally, I would like to acknowledge Sci-hub and all who contribute to it for creating such an essential resource for researches around the world. I would also like to thank Guarantors of Brain for travel grants and support and lastly, the OU and H2020 for funding this project and giving me the opportunity to make a contribution to the field of ageing and neuroscience.

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Conference proceedings

Oral presentations

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Age-induced changes in the neurovascular transcriptome’ November 2016, BBB Early careers Symposium, Dublin, Ireland.

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Ultrastructural and molecular age-associated changes in the female mouse neurovascular unit’ September 2017, BBB Early careers Symposium, Portsmouth, UK.

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Ultrastructural and molecular age-associated changes in the female mouse neurovascular unit’ November 2017, 12th CVB, Melbourne, Australia.

Frías-Anaya E, Figueiredo R, Wang J, Crea F, Winter P, Hawkes C, Romero IA. ‘Age-induced changes in gene expression and ultrastructure at the mouse blood-brain barrier’ June 2018, Barriers of the CNS GRS student conference, Boston, USA.

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Molecular and ultrastructural characterization of the ageing blood-brain barrier in female mice’ November 2018, BBB Early careers Symposium, Oxford, UK.

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Molecular and ultrastructural characterization of the ageing blood-brain barrier in female mice’ December 2018, RMS Beginners Competition, London, UK. *Best talk award.*

Poster presentations

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘Age-induced changes in the neurovascular transcriptome’ November 2016, BBB Early careers Symposium, Dublin, Ireland.

Frías-Anaya E, Figueiredo R, Crea F, Winter P, Hawkes C, Romero IA. ‘Age-induced changes in gene expression and ultrastructure at the mouse blood-brain barrier’ June 2018, Barriers of the CNS GRC, Boston, USA.

Frías-Anaya E, Hawkes C, Crea F, Romero IA. ‘The female blood-brain barrier in ageing: structural and transcript profile alterations’ June 2019, 12th CVB, Miami, USA.

Abbreviations

2D: two dimensional

3D: three dimensional

5mC: 5-methylcytosine

A β : amyloid- β

ABC: ATP-binding cassette

AD: Alzheimer's disease

ADAM10: Alzheimer disease-related A disintegrin and metalloprotease 10

AJs: adherens junctions

Ago2: argonaute 2

AQ4: aquaporin 4

ARE: antioxidant response element

BBB: blood-brain barrier

BEC: brain endothelial cell

bFGF: bovine fibroblast growth factor

BM: basement membrane

BSA: bovine serum albumin

Cldn-: claudin-

CGI: CpG island

CNS: central nervous system

DNMT/Dnmt: DNA methyltransferase

dsRNA: double stranded RNA

ECM: extracellular matrix

ER: oestrogen receptor

FAK/JNK: focal adhesion kinase/c-Jun N-terminal kinase

FBS: fetal bovine serum

FC: full cell

FDR: false discovery rate

FOV: field of view

GFAP: glial fibrillary acidic protein

GJs: GAP junctions

GSEA: gene set enrichment analysis

HBSS: Hank's balanced salts solution

ICAM-1: intercellular cell adhesion molecule 1
IGF: insulin-like growth factor
IFN: interferon
IL: interleukin
JACOP: junction-associated-coiled-coil protein
JAMs: junctional adhesion molecules
Log2FC: log2-fold change
LRP-1: lipoprotein-related protein-1
MAGUK: membrane-associated guanylate kinase-like proteins
miRNA/miR: micrRNAs
mRNA: messenger RNA
MACE-Seq: Massive Analysis of cDNA Ends sequencing
MMP: matrix metalloproteinase
NGS: next generation sequencing
NVU: neurovascular unit
OsO4: osmium tetroxide
p: passage
P/E2: progesterone/estradiol ratio
PB: phosphate buffer
PBS: phosphate-buffered saline
PE: permeability coefficient of endothelial monolayer
PECAM-1: Platelet endothelial cell adhesion molecule-1
PD: Parkinson's disease
PDGF β : platelet-derived growth factor- β
PDGFR- β : platelet-derived growth factor- β receptor
PFA: paraformaldehyde
piRNA: PIWI-interacting RNAs
Pgp: p-glycoprotein
pre-miRNA: precursor miRNA
pri-miRNA: primary miRNA
rhEGF: recombinant human epithelial growth factor
rhFGF: recombinant human fibroblast growth factor
RISC: RNA-induced silencing complex
RNA pol II: RNA polymerase II

RNA-Seq: RNA sequencing
ROS: reactive oxygen species
RT: room temperature
RT-qPCR: Reverse Transcription-Quantitative PCR
siRNA: small interfering/silencing RNA
SASP: senescence associated secretory phenotype
Shh: sonic hedgehog
Sirt1: sirtuin 1
SMC: smooth muscle cell
SMA- α : smooth muscle actin- α
TEER: transendothelial electrical resistance
TEM: transmission electron microscopy
TET: ten-eleven translocation proteins
TGF- β : transforming growth factor β
TJ: tight junction
TNF- α : Tumour necrosis factor α
TPM: tags per million
UTR: untranslated region
VCAM-1: vascular cell adhesion molecule 1
VE-cadherin: vascular endothelial cadherin
VEGF: vascular endothelial growth factor
ZO: zonula occludens

Chapter 1. General introduction

1.1 The Blood-Brain barrier

The first studies pointing to the existence of a barrier between the brain and the circulatory system were performed in the late XIX century and early XX century. Paul Ehrlich in 1885 and Edwin Goldmann in 1909, carried out a series of experiments that consisted in administration of vital dyes capable of staining living cells and tissues (i.e. trypan blue) into rats. Administrations were followed by examination of the dye distribution throughout the animal's body. When the dye was infused into the systemic blood circulation, Ehrlich observed that the brain remained unstained (Ehrlich 1885). Whereas when Goldmann performed the injection of the dye directly into the cerebrospinal fluid, it resulted in staining of the brain only (Goldmann 1909). There appeared to exist a barrier between the brain and the blood that would prevent the passage of the dye from the cerebral vessels into the brain. However, the term 'blood-brain barrier (BBB)' was not introduced until 1921 by Lina Stern and Raymond Gautier, who also performed detailed penetration studies with a wide range of molecules from blood into the brain (Stern and Gautier 1921). Additional experiments showing that intravenous administration of cholic acids and sodium ferrocyanide had no effects in the central nervous system (CNS), whereas intracerebral administration did, provided further evidence that brain capillaries modulate and regulate the passage of molecules from the blood stream into the brain and *vice versa* (Lewandowsky 1900).

Later studies (1960s) using electron microscopy techniques and horseradish peroxidase showed how this protein diffuses relatively fast across capillaries into peripheral tissues, whereas it would remain confined within the lumen of capillaries in the brain parenchyma for a longer period of time (Brightman et al. 1970). From these experiments it was concluded that the lack of permeability to peroxidase by brain and spinal cord microvessels was due to two main features present in the capillary brain endothelial cells (BEC): 1) the absence of channels for intracellular transport and 2) a low trafficking rate of pinocytotic vesicles. This assumption is complemented by the fact that inter-cellular tight junction (TJ) proteins are highly expressed in the brain endothelium, compared to peripheral endothelial cells, thereby providing the actual physical barrier properties of the whole anatomical structure. From this and later studies, it has been demonstrated that the BBB is localised at the level of the BECs (Kniesel and Wolburg 2000; Wolburg and Lippoldt 2002).

1.1.1 Vascularisation of the CNS

The CNS is highly vascularised, and as any other organ in the body its vascular network can be classified depending on the size and cell composition of the blood vessels. Following mainly the size criterion, cerebral vasculature is divided in macrocirculation and microcirculation. The main function of macrocirculation vessels is promoting the arrival and exit of blood into and out of the brain, whereas the microcirculation plays a role in regulating the blood flow and facilitating exchange of oxygen and nutrients (Kulik et al. 2008).

1.1.1.1 Macrovascular network in the CNS

Macrocirculation is comprised by cerebral blood vessels of higher size, which include the internal vertebral arteries that originate in the neck and fuse within the skull into the basilar artery. These arteries join at the base of the brain to form the circle of Willis, and from there arterial blood is carried by pial vessels, which are followed by penetrating arteries that finally reach the brain parenchyma (Zirak et al. 2010). The final aim of macrocirculation arteries is to perfuse the brain with oxygenated blood. Following gas exchange, deoxygenated blood is removed from the brain via cerebral veins that ultimately drain it into the transverse and sigmoid cranial sinuses (Hendrikse et al. 2005; Kılıç and Akakin 2007).

1.1.1.2 Microvascular network in the CNS

CNS microcirculation is comprised by smaller penetrating vessels. The microvascular network exists between the cerebral arterial and venous systems and includes arterioles, capillaries and venules, which are also known by the general term of microvessels. Arterioles (50 to 100 μm of diameter) arise from the larger penetrating arteries and are followed by pre-capillary arterioles (20 to 50 μm). Venules and post-capillary venules show a diameter similar to arterioles and pre-capillary arterioles and participate in the removal of deoxygenated blood (Macdonald, Murugesan, and Pachter 2010), these vessels present loose organization of cell to cell junctions, being the segment of the microvascular network where leukocyte extravasation preferentially occurs (Ge, Song, and Pachter 2005). Brain capillaries (less than 10 μm of diameter), are the site of gas exchange in-between arteriolar and venular systems, have the largest surface area of all CNS vessels, and are the ones in which the barrier characteristics,

including TJs, are highly prominent (Itoh and Suzuki 2012). As stated above, the BBB is located at the level of the BECs. With respect to its location within the neurovascular tree, the higher expression of a BBB is generally considered to exist at the level of microvessels, and that is the reason why most of the studies focus on them. However, bigger vessels such as cerebral arteries and veins also show barrier characteristics (Butt, Jones, and Abbott 1990; Ge et al. 2005; Mayhan and Heistad 1986).

1.1.1.3 *Microvessel heterogeneity*

Several studies have described segmental differences along the brain microvasculature. BECs have been shown to present gradual phenotypical and transcriptional changes depending on the level of the vascular hierarchy they are at, a phenomenon known as zonation (Vanlandewijck et al. 2018). Indeed, these differences at the level of BECs appear to be associated as well with structural, biochemical and functional changes among segments of the microvasculature. Zonation-specific differences include organization of TJs, rate of transcytosis, expression of enzymes such as Na⁺/K⁺ ATPase, expression of transporters such as P-glycoprotein (Pgp) and presence of mural cells (i.e. smooth muscle cells, pericytes). These parameters differ between arterioles, capillaries or venules, thus can also be used for classification (**Table 1**) (Saubaméa et al. 2012).

Table 1. Structural and functional differences between arterioles, capillaries and venules.

Presence of perivascular cells is indicated by Yes, No or Yes/No. Presence of BBB features is indicated by Yes/No/? (not specifically described). Relative expression of transporters, enzymes and transporters is indicated by +, ++ or +++, from lowest to highest expression; -, for absence; +/-, when available publications do not agree; ?, when not specifically described for a certain type of microvessels.

	Arterioles	Capillaries	Venules	References
Cells				
Smooth muscle cells	Yes	No	Yes/No	<i>Bechman et al., 2006; Vanlandewijck et al., 2018</i>
Pericytes	Yes/No	Yes	Yes	<i>Dalkara et al., 2011; Vanlandewijck et al., 2018</i>
Perivascular macrophages	Yes	Yes	Yes	<i>Bechman et al., 2006; Vanlandewijck et al., 2018</i>
BBB features				
Tight Junctions (TJs)	Continuous TJs/?	Highly expressed TJs	Loosely organized TJs	<i>Dahl, 1973; Simionescu et al., 1976; Ge et al., 2005</i>
Permeability for BBB markers	?	No	Yes	<i>Mayhan et al., 1985; Bechman et al., 2006</i>
Astrocytic end-foot sheath	No	Yes	No	<i>Bechman et al., 2006; Ge et al., 2005</i>
Transporters/Enzymes/Receptors				
P-glycoprotein	?	+++	?	<i>Virgintino et al., 2002; Golden & Pardridge, 2000</i>
Na ⁺ /K ⁺ - ATPase	+++	+	+	<i>Ge et al., 2005</i>
Transferrin receptor (TFRC)	-	+++	+/-	<i>Kalaria et al., 1992; Vanlandewijck et al., 2018</i>
Alkaline phosphatase	+++	+++	+	<i>Ge et al., 2005</i>
Mg ²⁺ - ATPase	+++	+	+	<i>Ge et al., 2005</i>
5' -nucleotidase	+++	+	+	<i>Ge et al., 2005</i>
γ-Glutamyl Transpeptidase (GGTP)	?	+++	?	<i>Wolff et al., 1992; Hanigan & Frierson, 1996</i>
Bidirectional horseshoe peroxidase transp.	+++	+	+	<i>Ge et al., 2005; Sheikov et al., 2006</i>

1.1.2 Components of the BBB

As discussed above, the unique expression of TJ proteins by BECs confers the barrier properties and selectivity of the BBB itself. The BBB is essential for the separation between brain parenchyma and circulatory system. Therefore, it contributes to the proper functionality and tight homeostasis regulation of the CNS (Popescu et al., 2009).

Although extremely important, BBB properties are not only due to the BECs. Blood vessels include BECs, mural cells (pericytes specifically) that sit on the basal surface of the endothelial monolayer and are embedded in a basement membrane (BM), sheathed by the astrocytic end-feet (**Figure 1**). These cellular and acellular components play a role in inducing and maintaining the BBB properties. In addition, microglia, neurons and oligodendrocytes have also been shown to contribute to this barrier (Banerjee and Bhat 2007; Seo et al. 2014). Thus, the group of cells that form the vessel, including the TJ-expressing BECs, is known as the neurovascular unit (NVU) (Daneman and Prat 2014). Although NVU refers to the different interacting cell types, and the BBB to the actual barrier at the endothelial level, both terms are usually exchangeable in literature.

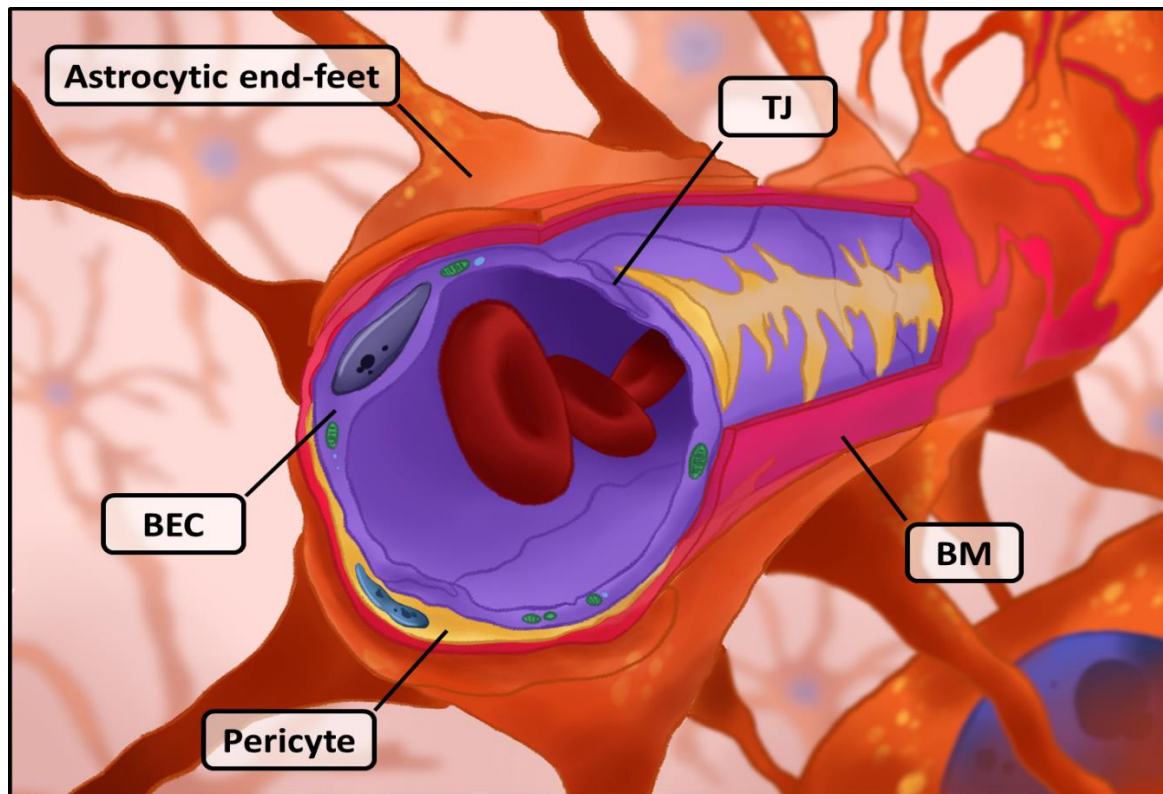


Figure 1. Main components of the BBB at capillary level.

Brain endothelial cells (BECs) are distributed in continuous layers forming the blood vessels walls. They establish tight junctions (TJs) with neighbouring BECs to limit paracellular transport across the endothelium. Pericytes surround the BECs, sharing a common basement membrane (BM) with them. Astrocytic end-feet ensheath the microvessel and connect vascular and neural components. (*Diagram by Eduardo Frías Anaya*).

1.1.2.1 Brain endothelial cells (BECs)

Blood vessels in the brain are formed by a continuous layer of BECs which are considered the first physical barrier in order to maintain brain homeostasis. This specialty is due to unique properties different from all other endothelial cells in the body (Sá-Pereira, Brites, and Brito 2012).

Unlike endothelial cells from peripheral circulation, BECs are non-fenestrated, present low pinocytotic activity, their surface is negatively charge, thereby limiting solute exchange between brain and blood, and their number and volume of mitochondria is also higher, providing energy for selective enzymes and active transport of certain solutes (Cardoso, Brites, and Brito 2010). Mainly due to the expression of highly organized TJ proteins, BECs also exhibit limited paracellular (i.e. between cells) flux of hydrophilic molecules across the

endothelium and have a high electrical resistance (average $3000 \Omega \cdot \text{cm}^2$, *in vivo* conditions) (Ballabh, Braun, and Nedergaard 2004; Bradbury 1993).

1.1.2.2 Pericytes

Pericytes are located adjacent to endothelial cells on the abluminal (brain-facing) side of capillaries, precapillary arterioles, postcapillary venules and collecting venules. Pericytes are embedded within the endothelial-astrocyte BM and are able to send cellular projections that cover substantial parts of the vascular circumference (Balabanov and Dore-Duffy 1998). Pericyte recruitment onto vessels during development depends on the relationship between platelet-derived growth factor- β (PDGF β), secreted by endothelial cells and its receptor PDGFR β , which is expressed in pericytes (Armulik, Abramsson, and Betsholtz 2005; Hellström et al. 1999). The close relationship between BECs and pericytes is further demonstrated in areas where the BM is absent and via the formation of ‘peg-and-socket’ contacts that consist of membrane invaginations from both cell types and contain junctional contacts such as TJs, but also GAP and adherens junctions (Gerhardt and Betsholtz 2003). This way, pericytes are thought to facilitate and integrate cell communication in the BBB (Dalkara, Gursoy-Ozdemir, and Yemisci 2011). CNS pericytes are also important for control of the growth and migration of BECs in *de novo* formation of microvessels as well as for maintaining the integrity of brain capillaries through signalling pathways (e.g. Notch pathway) (Stratman and Davis 2012; Wang et al. 2013). They also regulate TJ formation and paracellular permeability of the BBB via expression of growth factors. Indeed, transforming growth factor β (TGF- β) released by pericytes was shown to increase barrier tightness in mouse primary BECs (Dohgu et al. 2005), while pericyte-derived secretion of angiopoietin-1 has been reported to induce expression of TJ proteins (e.g. occludin) in cultured rat BECs (Hori et al. 2004). Similarly, it has been reported how interactions between pericytes and astrocytes promote polarization of astrocytic end-feet and consequently the ensheathment of blood capillaries, contributing to BBB stability (S. Liu et al. 2012). Additionally, several studies have reported the expression of pericytic contractile proteins, which suggests that pericytes contribute to controlling capillary diameter and modulating cerebral blood flow at the level of capillaries, in response to changes in neuronal activity (Cai et al. 2018; Hall et al. 2014; Peppiatt et al. 2006).

1.1.2.3 Basement membrane (BM)

The endothelial BM, or basal lamina, within which pericytes are embedded, is located on the abluminal side of the blood vessel and separates pericytes from BECs and astrocytic end-feet (Yurchenco and Patton 2009). BECs, pericytes and astrocytes all contribute to the formation and maintenance of the BM (Abbott, Revest, and Romero 1992). Capillaries contain an endothelial BM and an astrocytic BM that are in close contacts and often appear as one. This non-cellular component is a 30 to 40 nm thick layer comprised mainly of collagen type IV (important for the integrity of small vessels), laminins (mediate attachment between BM and cells), heparin sulphate proteoglycans like perlecan (provides a negative charge) and glycoproteins such as fibronectin (Sá-Pereira et al. 2012).

The extracellular matrix (ECM) of the BM contains proteins that provide an anchor for the endothelium via interaction of laminins and integrin receptors located in the endothelial cells, as well as proteins that regulate expression of TJ proteins, which suggests contribution to barrier properties (Engelhardt 2011). Accordingly, disruption of the ECM has been associated with increased BBB permeability (Hawkins and Davis 2005). Also, the interaction between BM and astrocytes has been proved to promote the specific localisation of several membrane proteins (e.g. dystrobrevins and dystroglycans) in the astrocytic end-feet anchored to the BM, thereby contributing in turn to the contact and communication between astrocytes and brain endothelium (Abbott, Rönnbäck, and Hansson 2006).

1.1.2.4 Astrocytes

As a component of the BBB, astrocytes have been shown to play an important role in the induction and maintenance of the barrier properties in the BECs, including overexpression of proteins forming TJ complexes (Bauer and Bauer 2000). Astrocytes send specialized processes, known as end-feet, to closely ensheath the brain capillaries on their outer surface, ultimately covering the BM, BECs and pericytes (Newman 1986). Astrocytic end-feet promote the interaction between the vascular and the neural components, and express specific membrane transporters such as Aquaporin 4 (AQ4, water channel) or the potassium channel Kir4.1, while also release factors that regulate the expression of several proteins and barrier properties in the BEC, including TGF- β , sonic hedgehog (Shh), bovine fibroblast growth factor (bFGF) or angopietin-1 among others (Obermeier, Daneman, and Ransohoff 2013). These astrocyte-derived factors have been shown to modulate expression levels of TJ proteins or

transport systems in the endothelium, thereby contributing to BBB stability (Haseloff et al. 2005; Wolburg et al. 1994).

1.1.2.5 Smooth muscle cells (SMCs)

SMCs are present as mural cells on the abluminal side of endothelial cells in arteries, veins and pre-/post-capillary vessels (Bechmann, Galea, and Perry 2006), whereas capillaries lack the continuous layer of SMCs (Peppiatt et al. 2006). Some of the proteins and markers that define the SMCs include smooth muscle actin α - (SMA- α), SM-myosin or desmin (Hirschi, Rohovsky, and D'Amore 1998). SMCs contribute to the maintenance and regulation of blood pressure and flow via their contractile ability (Hughes and Chan-Ling 2004). Interestingly, SMCs are developmentally related to other type of mural cells, pericytes, which also appear to play a role in contraction and blood flow at the capillary level (Rhodin 1968). Also, as described above for pericytes, SMCs are recruited by endothelial and have been shown to participate in vasculature development and contribute to vessel function during the life of the individual including vascular stabilization, matrix formation, and BBB properties (Korn, Christ, and Kurz 2002).

1.1.2.6 Perivascular microglia

Microglial cells are originally derived from leptomeningeal mesenchymal cells that differentiate into microglia when entering the brain (Bechmann et al. 2006). Alternatively, circulating monocytes also provide an important source of perivascular microglia when infiltrating the brain (Bechmann et al. 2005). Perivascular microglia are therefore macrophages in close contact with brain blood vessels and have been reported to express markers involved in antigen recognition and presentation (Fabriek et al. 2005). These observations suggest that microglial cells participate in regulating perivascular inflammation in the brain, which together to their strategic position in the BBB allows them to contribute to the control of immune responses in the CNS (Correale and Villa 2009). In addition, perivascular microglia have been shown to participate in phagocytosis of BECs or subcellular endothelial-derived particles in situations of brain injury or ischemia (Jolivel et al. 2015).

1.1.3 Structural and molecular organization of junctional complexes in brain microvasculature

It has already been established that the barrier properties of the BBB are conferred by the expression of and interaction between junctional proteins. These junctional complexes include mainly TJs, adherens junctions (AJs) and GAP junctions (GJs). TJs in the brain endothelium are more complex and its protein constituents are highly expressed when compared to the rest of the body, contributing to the barrier phenotype (Ge et al. 2005). TJs are located at the limit between the apical and basolateral domains of the BEC surface, forming continuous belts of intermingled proteins (**Figure 2**). Three types of transmembrane proteins have been described to participate in the formation of TJs, including claudins, occludin and junctional adhesion molecules (JAMs) (Bechmann et al. 2006). In addition, adaptor and scaffolding proteins present in the cytoplasm such as zonula occludens 1, 2 (ZO-1, -2), junction-associated-coiled-coil protein (JACOP, also known as para-cingulin) and cingulin, allow interactions between TJs and the actin cytoskeleton interactions, as well as signal transduction (Balda and Matter 2009; Brightman and Reese 1969). TJs interact with AJs, which also contribute to cell-cell contact between BECs. AJs comprise vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecules 1 (PECAM-1, or CD31), they are linked to the cytoskeleton via interaction with catenins (Daneman and Prat 2014). Apart from all this intricate structure of junctional protein complexes, BECs also connect to each other through GJs, formed mainly by connexin channels that promote direct intercellular communication. Ultimately, BECs are anchored to the underlying BM via integrins and dystroglycans, which interact with ECM proteins (Zhao et al. 2015).

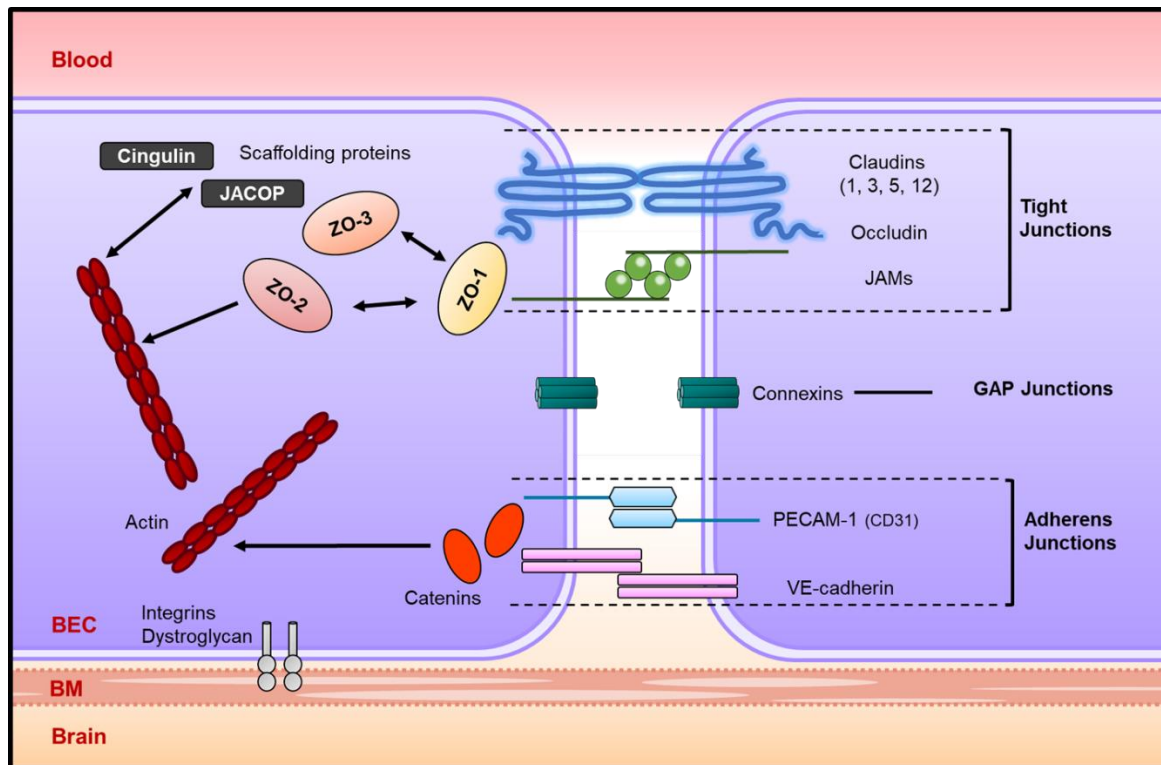


Figure 2. Molecular organization of TJs at the BBB.

TJs are mainly comprised by claudins, occludin and JAMs. Occludin and claudins share a common structure and are essential for BEC inter-membrane contacts between BECs as well as TJ establishment and regulation. JAMs are members of the immunoglobulin superfamily and participate in TJ stability and leukocyte adhesion and migration across the BBB. Additionally, TJs are intertwined with AJs, which are mainly comprised by PECAM-1/CD31 and VE-cadherin. PECAM-1 controls the location of VE-cadherin and is involved in vascular integrity, whereas VE-cadherin is involved in the formation and strength of junctional contacts. BECs also express GJs comprised by connexins that mediate direct communication between neighbouring cells. These membrane proteins interact with cytoplasmic adaptor proteins, including ZO-1, -2 and -3 which are essential for the assembly and scaffolding of TJs and connect the junctional protein to the cytoskeleton via cingulin or JACOP proteins. Catenins also participate in the stabilisation and anchoring of AJ proteins. In addition, integrins and dystroglycan anchor BECs to the extracellular matrix if the BM (*Diagram by Eduardo Frías Anaya*).

1.1.3.1 Occludin

Occludin is a 60-65 kDa protein that is highly expressed in endothelial cells of the CNS and was the first integral transmembrane protein identified as part of the TJs in BECs; occludin is found in cell contact sites at the basolateral surface of the cell, where it establishes homophilic interactions with other occludins in neighbouring BECs and also with claudins, as a way of promoting TJ maintenance (Kniessel and Wolburg 2000). Occludin structure includes

a terminal domain that links to the actin cytoskeleton via scaffolding proteins such as ZO-1 and ZO-2 by binding to PDZ domain, which stabilizes its localisation in the plasma membrane (Feldman, Mullin, and Ryan 2005). Although several studies suggest occludin is not essential for the formation of TJs (Furuse et al. 1998), reduced expression of occludin is associated to BBB dysfunction (Brown et al. 1999). Interestingly, some studies have shown occludin phosphorylation to be a key regulator in TJ permeability (Hirase et al. 2001), especially in size-selection diffusion in the case of epithelial cells (Balda et al. 1996, 2000).

1.1.3.2 Claudins

Claudins are integral membrane proteins of 20-27 kDa that have similar membrane topography to occludin but not sequence homology, and are involved in TJ formation in various tissues (Morita et al. 1999). Most claudins present tissue specific expression, brain tissue expresses mostly claudin -5, but also claudin-1, -3 and -12 (Cldn-5, -1, -3, -12) (Liebner, Fischmann, et al. 2000; Liebner, Kniesel, et al. 2000). Due to its higher levels in the brain endothelium, claudin-5 has been suggested to be the major constitutive claudin at the BBB (Ohtsuki et al. 2007). Nevertheless, other studies have shown how the BBB remains ultrastructurally normal in claudin-5 knock-out mice, followed by an increase in claudin-1 (Berndt et al. 2019). These observations suggest a far more relevant role for other claudins apart from claudin-5 than originally thought. Claudins establish homophilic and heterophilic interactions with other claudins in adjacent BECs in order to promote TJ sealing (Furuse, Sasaki, and Tsukita 1999). Additionally, claudin carboxyl-terminal domains bind to cytoplasmic ZO proteins in a similar way to occludin (Itoh et al. 1999).

1.1.3.3 JAMs

JAMs are 40 kDa glycoproteins, members of the immunoglobulin superfamily whose structure includes a short intracellular carboxyl-terminal domain by which they interact with ZO-1 via a PDZ domain (Martín-Padura et al. 1998; Wolburg and Lippoldt 2002). Extracellularly, these adhesion proteins establish homophilic or heterophilic interactions with adjacent cells contributing to TJ structure and have also been reported to participate in modulating leukocyte transendothelial migration (Del Maschio et al. 1999; Ostermann et al. 2002).

1.1.3.4 *PECAM-1/CD31 and VE-cadherin*

PECAM-1 is a 130 kDa transmembrane glycoprotein member of the immunoglobulin superfamily, highly expressed on the basolateral surface of endothelial cells (Newman et al. 1990). PECAM-1 extracellular domain mediates homophilic interactions with adjacent cells and by interaction with leukocytes it plays a role in their transendothelial migration (Liao et al. 1995; Muller et al. 1993; Sun et al. 1996). On the other hand, PECAM-1 cytoplasmic domain phosphorylation mediates regulation of signalling complex assembly and interaction with the cytoskeleton (Ilan et al. 2000). Regarding its role in adherens junctions, PECAM-1 controls the subcellular location of VE-cadherin and β -catenin, which contributes to maintaining barrier integrity and permeability (Park et al. 2010; Wimmer et al. 2019). Also, PECAM-1 is involved in vascular integrity, acting as an important vascular mechanosensor in angiogenesis and vessel remodelling (Chen and Tzima 2009; Osawa et al. 2002).

Cadherins are cell adhesion molecules involved in the establishment and maintenance of junctional contacts (Suzuki, Sano, and Tanihara 1991). There are several cadherins expressed by endothelial cells but the only specific one is VE-cadherin, a 140 kDa transmembrane protein located in the basolateral cell surface (Gotsch et al. 1997; Pulous et al. 2019). VE-cadherin extracellular domain mediates homophilic Ca^{2+} -dependent interactions with neighbouring endothelial cells contributing to barrier properties (Ali et al. 1997). Furthermore, the cytoplasmic tail contains catenin-binding sites (for p120, α -catenin, β - and γ -catenin) that mediate VE-cadherin connection to the actin cytoskeleton (Noren et al. 2000). Interestingly, VE-cadherin and catenins together are suggested to function as an early-recognition mechanism between BECs, being involved in the formation of intercellular junctions (Vincent et al. 2004; Vorbrodt and Dobrogowska 2004).

1.1.3.5 *Tight and adherens junction-associated submembranous proteins*

Junctional proteins form intracellular connections with cytoplasmic ZO-1, -2 and -3, members of the membrane-associated guanylate kinase-like proteins (MAGUK) family. These proteins play a role in the assembly and scaffolding of TJs, connecting them via cingulin to the actin cytoskeleton and also working as support for signal transduction proteins (Huber, Egleton, and Davis 2001). ZO proteins show structural homology, and all contain PDZ

domains, specific for the interaction with cytoskeleton and TJ proteins (Itoh et al. 1999). Indeed, ZO proteins interaction with claudins promotes formation of functional TJ strands (Umeda et al. 2006). Moreover, these proteins participate in transduction of several signals, thereby influencing cellular processes such as establishment of cell polarity, adhesion complexes or cell survival (González-Mariscal, Betanzos, and Ávila-Flores 2000). Interestingly, ZO-1 has also been reported to modulate VE-cadherin role in cell tension and cytoskeleton organization, and also has been linked to barrier formation (Tornavaca et al. 2015). In fact, lack or decreased expression of ZO proteins leads to mislocalisation of junctional adhesion complexes, higher barrier permeability and BBB disruption (Shimajima et al. 2008).

The catenins are cytoplasmic anchor proteins of the Armadillo family that mediate the linkage of cadherins to the cytoskeleton (Ozawa and Kemler 1992). BECs mostly express α -catenin, β -catenin, γ -catenin (plakoglobin) and p120, which share multiple repeats of the Armadillo sequence (Peifer et al. 1992). These proteins bind to VE-cadherin through its cytoplasmic tail and to α -catenin, which interacts with actin-binding proteins such as α -actinin, ZO-1 and others (Itoh et al. 1997; Knudsen et al. 1995; Rimm et al. 1995). VE-cadherin interaction with catenins modulate junctional stabilisation and anchoring and barrier permeability (Dejana, Orsenigo, and Lampugnani 2008; Liebner, Gerhardt, and Wolburg 2000). In addition, catenins are also crucially involved in cell signal transduction including Wnt canonical signalling pathway, a role that has been shown to be fundamental in CNS vascularization and BBB formation and maturation (Tran et al. 2016).

1.1.4 Functions of the BBB

In the CNS, neurons communicate via chemical and electrical signals, therefore a regulated microenvironment and a properly maintained homeostasis are required for a reliable neural function. The presence of the BBB allows the brain to tightly regulate the entry of selected cells, proteins and molecules from the circulation and maintain CNS homeostasis (Aschner and Aschner 1990; Gee and Keller 2005).

1.1.4.1 *Physical barrier and selective transport systems*

Pathways across the BBB are either transcellular (across the cell body, controlled by specific transporters) or, more limited, paracellular (in-between BECs, depending on TJs). Indeed, the presence of junctional protein complexes, as well as a lack of fenestrations in the brain microvessel endothelium reduces the paracellular passage of ions and other small hydrophilic molecules between cells, therefore inducing an actual physical barrier that separates the CNS from the circulatory system and promoting mostly transcellular mechanisms (Abbott 2013; Haseloff et al. 2015). BECs are permeable to small lipophilic molecules and gases such as O₂, CO₂ and ethanol (Friis, Paulson, and Hertz 1980; Paulson 2002). Smaller molecules may cross the BBB by receptor-mediated transcytosis or endocytosis, although 98% of all small molecules do not freely move across the barrier (Mikitsh and Chacko 2014; Pardridge 1995). Large molecules such as proteins and certain peptides including glucose, amino acids and nucleosides, get into the brain by regulated and selective transport systems (Boado et al. 1999; Kalaria et al. 1988). Accordingly, the BBB also regulates both recruitment and entry of immune cells that participate in the surveillance of the normal CNS immune environment (Loeffler et al. 2011). Additionally, the BBB promotes the removal of waste products and other solutes out of the brain, using efflux pumps to regulate the passage (Hagenbuch, Gao, and Meier 2002; Ito et al. 2013).

Differential expression of specific solute carriers and enzymes determines cellular polarity in the BECs, with membrane proteins and transporters being asymmetrically distributed on the luminal (blood-facing) and abluminal sides (Betz, Firth, and Goldstein 1980). Therefore, transporters present in the BBB include transporter system of monocarboxylic acids, amino acid transporters such as L1 transporter or γ -glutamyl transpeptidase, GLUT-1 glucose carrier or transferrin receptor (Ge et al. 2005; Janzer 1993). Specific ion channels and transporters expressed at the BBB also help to maintain physiological and metabolic conditions of the brain. For instance, the Na⁺/K⁺ – ATPase actively transports Na⁺ and K⁺ across the BBB, while Mg²⁺ is also actively transported by ATPase-related enzymes (Harik 1986; Serlin et al. 2015). On the other hand, the BBB also expresses several pumps aimed to actively protect the brain environment. For example, ATP-binding cassette (ABC) transporters located on the plasma membrane of BECs act as efflux pumps that protect the CNS from the entry of neurotoxicants, but also limit the access of therapeutic compounds into the parenchyma (Miller 2010). Amongst these ABC transporters, Pgp is highly expressed in the luminal surface of the

brain capillary endothelium and interacts with a large number of drugs, contributing to their active exclusion from the BECs back into the blood stream (Potschka and Löscher 2001; Vogelgesang et al. 2012).

1.1.4.2 CNS immune surveillance

The brain has traditionally been considered immune privileged in healthy conditions due to the lack of classical lymphatic systems, low levels of major histocompatibility complex molecules and the unique presence of the BBB (Kleine and Benes 2006). However, despite the strict separation between the circulatory system and the CNS, it has been shown that both the brain and the spinal cord are under continuous immune surveillance to detect and eliminate potential threats. The primary immune agents, are microglia and circulatory immune cells (i.e. lymphocytes) from the blood that also contribute to this defence and the maintenance of brain homeostasis in absence of inflammation (Ousman and Kubes 2012). For instance, blood-borne macrophages located at the perivascular spaces of the brain vasculature have also been reported to contribute to CNS immune surveillance (Bechmann et al. 2001).

1.1.5 Interactions between cells and ECM at the BBB

The integrity of the barrier properties of the brain endothelium also depends on the interactions with the ECM and the BM, since adhesion to the ECM is required for an appropriate stabilization of the whole BBB structure, with the BM also acting as a key outer barrier to the mature BBB (Menezes et al. 2014; Reed et al. 2019). Integrins and dystroglycans are two main adhesion proteins involved in the cell/ECM interactions at the BBB. Integrins are transmembrane glycoprotein non-covalently-linked $\alpha\beta$ heterodimers that bind specific ECM ligands, activate different signal transduction mechanisms, and are normally expressed in all cell types that comprise the BBB. Their activation affects processes such as survival, proliferation, differentiation and migration (Hynes 1992). Interestingly, these effects are mediated by signalling pathways, including focal adhesion kinase/c-Jun N-terminal kinase (FAK/JNK), Ras/ERK (MAP kinase) or small GTPases like Rho, which may end up activating growth factors (Giancotti and Ruoslahti 1999; Miranti and Brugge 2002). Dystroglycan, is a non-integrin single $\alpha\beta$ heterodimeric (α , extracellular; β , intracellular) transmembrane receptor, involved in the linkage of cytoskeleton with the ECM, and is mainly expressed in perivascular astrocytes and BECs (Zaccaria et al. 2001). The intracellular β -dystroglycan subunit binds the

cytoskeleton, via interaction with the extracellular α -dystroglycan subunit, to specific ECM ligands such as laminins, perlecan or agrin, thereby promoting anchorage of BECs and the astrocytic end-feet to the BM (Moukhles and Carbonetto 2001; Del Zoppo et al. 2006).

1.2 The ageing BBB: process and highlights

The process of ageing is an inevitable, continuous and time-dependent decline in tissues and organs with progressive loss of function, decreasing fertility and increasing mortality (Balcombe and Sinclair 2001). The brain, as any other organ in the body is affected by ageing at both cellular and molecular level, with the hippocampus and the cerebral cortex amongst the most affected regions (Anderton 2002). Ageing in the CNS has been reported to include changes in gene and protein expression, abnormal protein accumulation or mitochondrial dysfunction (Brunk and Terman 2002; Liang et al. 2007; Trojanowski and Mattson 2003). These age-related changes have been implicated in promoting neurodegeneration, declined cognitive function, reduced cerebral blood flow and vasculopathies such as stroke (Cabeza et al. 2018; Desjardins et al. 2014; Gupta et al. 2012; Wyss-Coray 2016). Accordingly, the BBB has been described to undergo several alterations in function and structure during ageing (Krause, Fautsmann, and Dermietzel 2002; Pelegrí et al. 2007).

As previously discussed, the BBB plays a critical role in maintaining the homeostasis of the brain. Alterations and declined integrity of the BBB have been implicated in a variety of age-related neurodegenerative conditions, such as vascular cognitive impairment, Alzheimer's disease (AD) and Parkinson's disease (PD) (Marques et al. 2013; Zlokovic 2008). As the percentage of individuals over 60 years has increased from 9.2% in 1990 to 11.7% in 2013 and is projected to be 21.1% by 2050 (Sander et al. 2015), understanding the mechanisms that underlie age-related changes in the BBB in health and disease is of increasing importance (Aunan et al. 2016).

1.2.1 Breakdown of the BBB during ageing

During ageing, the cerebrovasculature undergoes several pathophysiological changes that lead to its dysfunction. For instance, cerebrovascular ageing is characterised by alterations in blood flow dynamics which induce changes in the expression of mechanosensitive genes that in turn promote vascular remodelling or pro-atherogenic modifications in the vessel wall

(Ungvari et al. 2010). Indeed, cerebral microvessels have been reported to become tortuous and show severe changes such as increased wall stiffness or decreased density, that contribute to a decline in cerebrovascular efficiency and cerebral blood flow (Riddle, Sonntag, and Lichtenwalner 2003). Interestingly, as cerebrovascular efficiency falls, the brain also suffers from impaired transport of essential elements like glucose or oxygen (Peters 2006; Yamazaki et al. 2016). In addition, aged cerebrovasculature might also undergo process of neuroinflammation and oxidative stress (Enciu, Gherghiceanu, and Popescu 2013). BBB alterations have also been observed to vary depending on the brain region in both aged humans and rodents, contributing to the complexity of the ageing process (Goodall et al. 2018; Hawkes et al. 2013).

BBB dysfunction during ageing has been related to numerous alterations in the endothelium, at both cellular and molecular levels (**Figure 3**). These age-related changes include:

- Increased BM thickness due to the accumulation of ECM components such as collagen IV, laminin and other proteoglycans, which in turn induces an increase in stiffness of the vessel wall (Candiello, Cole, and Halfter 2010). Higher BM thickness and stiffness may have detrimental effects on cerebral blood flow and clearance mechanisms across the BBB that may contribute to cognitive decline and neurodegeneration (Ito et al. 2013).
- Weakening of TJ protein structures, which may lead to higher BBB permeability (Montagne et al. 2015). For example, levels of TJ proteins such as occludin, Cldn-5 and ZO-1 have been reported to decrease in aged rodent brain compared to their young counterparts (Elahy et al. 2015; Mooradian, Haas, and Chehade 2003; Stamatovic et al. 2019). Also, previous studies have shown an age-induced increase in matrix metalloproteinase (MMP) expression, including MMP-9 and MMP-2, in the aged mice upon brain injury, which contributes to the cleavage of ZO-1 and claudin-5 and the increase in barrier permeability (Lee et al. 2012). Additionally, MMP-mediated disruption of TJ protein complexes was enhanced by increased levels of cytokines (e.g. Tumour necrosis factor α , TNF- α) in previous studies using an *in vitro* dynamic BBB model with rat BEC cells (Krizanac-Bengez et al. 2006). Similarly, TJ disruption may also be

worsened by exposure to reactive oxygen species (ROS) derived from oxidative stress, which may induce redistribution and loss of occludin or claudin-5 as reported in rat BECs *in vitro* (Schreibelt et al. 2007).

- BBB transport systems have been described to be altered with ageing, including enhanced pinocytosis or decreased expression of glucose transporter, insulin receptor or low density lipoprotein-related protein-1 (LRP-1), as observed in the ageing human and rodent brain (Cholerton, Baker, and Craft 2011; Mooradian et al. 1991; Ramanathan et al. 2015). Similarly, Pgp expression has been reported to decrease in the ageing BBB, although only in humans (Bartels et al. 2009). In any case, these changes lead to a severe imbalance in brain vasculature nutrient uptake/waste elimination function (Silverberg, Messier, et al. 2010; Silverberg, Miller, et al. 2010).

- Mitochondrial dysfunction is another prominent feature in the process of ageing in general and at the BBB in particular. Previous studies have described the age-related decrease in mitochondrial number and impairment in the BBB of several species including human, mouse and monkey, mostly related to oxidative stress and pro-inflammatory processes (Burns et al. 1979; Sure et al. 2018). As stated above, oxidative stress might contribute to vascular and BBB disruption. In fact, age-induced increase in production of ROS has been linked to endothelial cell dysfunction (Brandes, Fleming, and Busse 2005).

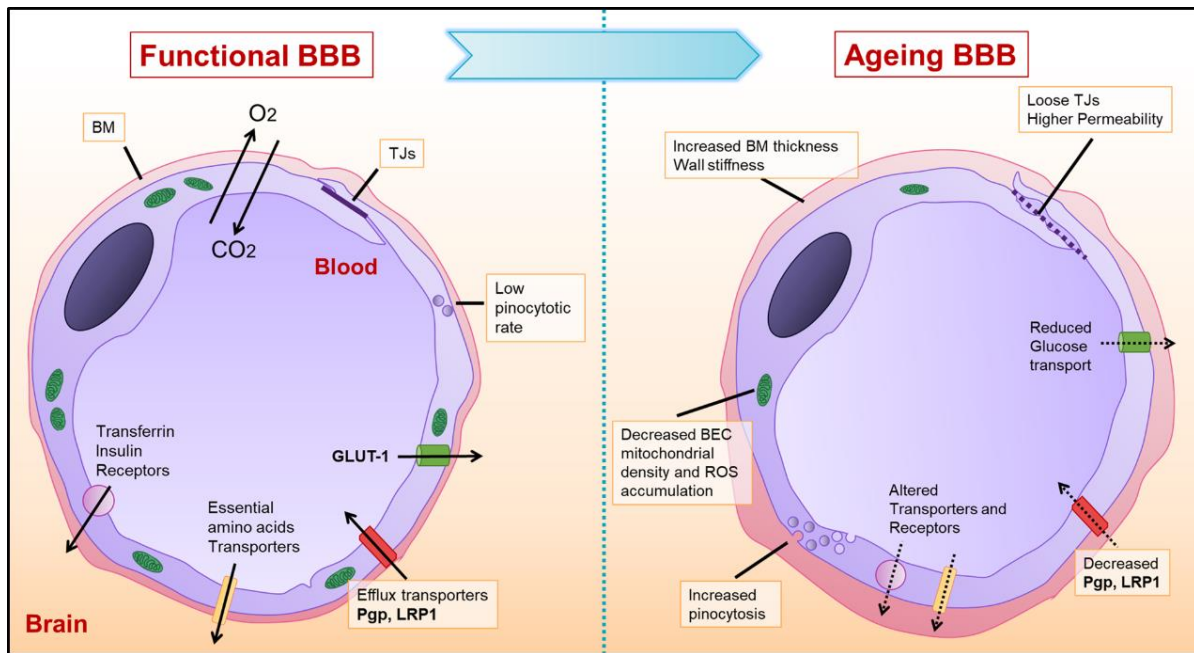


Figure 3. BBB cellular and molecular alterations in ageing.

During ageing, several alterations can be observed at structural and functional levels as the breakdown of the BBB progresses. A prominent feature is the increase in BM thickness due to the accumulation of extracellular matrix components. TJ protein structure becomes loose and weaker, which may lead to increased permeability. Transport systems are modified in the ageing BBB, including increased pinocytotic vesicles trafficking through the endothelium, as well as altered expression of transporters and membrane proteins such as glucose and insulin transporters and Pgp. In addition, there is a decrease in mitochondrial density and accumulation of ROS that contribute to oxidative stress and neuroinflammation, worsening the features of the ageing BBB. (*Diagram by Eduardo Frías Anaya*).

Several of these age-related changes at both cellular and molecular level appear to correlate with changes in gene expression to a certain degree (Osgood et al. 2017; Stamatovic et al. 2019). Therefore, connecting age-related changes in structure and function with concomitant alterations in gene expression profiles might shed light on the mechanisms that promote BBB dysfunction during ageing.

1.3 Gene expression modulation in the brain endothelium

As previously discussed, endothelial cells across the body present phenotype heterogeneity, thus BECs show specific characteristics different to endothelial cells in the kidney or the liver for example (Vanlandewijck et al. 2018). This heterogeneity can be

explained as the endothelium meeting the needs of the underlying tissue, although it could also be related to specific combinations of input/output signals between the endothelium and the extracellular environment in conditions such as injury, barrier disruption or ischemia (András et al. 2005; Jin et al. 2000). These signalling pathways are often triggered on the cellular surface and conclude at the gene transcription level (Y. Chen et al. 2003), therefore the microenvironment in which endothelial cells are immersed will promote the expression of genes that vary depending on the tissue, and in the case of BECs, they are influenced by neighbouring cells such as astrocytes and pericytes but also by microglia, neurons and blood-borne cells (Gaengel et al. 2009; Haseloff et al. 2005; Hordijk 2006).

Changes in BEC transcription profile may also involve epigenetic modifications (Hu et al. 2006; Zhao et al. 2016). Epigenetics is defined as the interaction between environmental factors and the genome, that results in modified gene expression or phenotypical characteristics without changes in the actual DNA sequence (Jones and Takai 2001; Reik, Dean, and Walter 2001). Indeed, epigenetic mechanisms are chromatin-based modifications that include DNA methylation, histone modifications and RNA-based modulation (e.g. microRNA (miRNA)-mediated regulation), which ultimately have an effect on gene expression (Matouk and Marsden 2008). Amongst them, miRNA- and DNA methylation-dependent mechanisms will be described in more detail, as they have been recently demonstrated to play a crucial role in ageing (Harries 2014; Horvath and Raj 2018).

1.4 MiRNAs as regulators of gene expression

1.4.1 MiRNAs: definition

MicroRNAs (miRNAs) are a classification of small RNAs (20-30 nucleotides) that can interact with Argonaute family proteins (Ago proteins) along with small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs, bind the PIWI clade of the Ago family) (Grillari and Grillari-Voglauer 2010). Amongst them, miRNAs are a dominating class of small RNAs first discovered during the late 1980s in the nematode *Caenorhabditis elegans* (Ambros 1989). They are highly conserved non-coding RNAs of ~22 nucleotides in length that regulate the expression of complementary messenger RNAs (mRNAs) by base-pair binding to the 3' untranslated region (UTR), which typically promotes silencing of gene by blocking mRNA

translation or promoting mRNA degradation, among other mechanisms (Ambros 2004; Ha and Kim 2014). Throughout the genome, miRNA genes are located in intergenic regions but also in defined transcription units, including exons and introns of protein- and non-coding genes as well as UTR regions (Rodriguez et al. 2004).

1.4.2 *MiRNA biogenesis*

All miRNA genes share a stem-loop precursor RNA structure and their transcription is typically mediated by RNA polymerase II (RNA pol II) (Lee et al. 2004) (**Figure 4**). Intergenic miRNAs are mainly transcribed through the canonical pathway as primary miRNA (pri-miRNA) transcripts that are typically spliced, capped and polyadenylated in a similar way to what happens with protein-coding mRNAs (Cai, Hagedorn, and Cullen 2004). Furthermore, during or subsequent to transcription, pri-miRNAs are processed by the endonuclease Drosha in interaction with the RNA-binding protein DGCR8, which is necessary for the cleavage of the pri-miRNA into an imperfect stem-loop precursor miRNA (pre-miRNA) of ~70 nucleotides with a hairpin structure (Han et al. 2004). Following this Drosha-mediated processing, the pre-miRNAs are exported from the nucleus to the cytoplasm by a Ran-GTPase transporter, Exportin 5 (Bohnsack, Czaplinski, and Gorlich 2004).

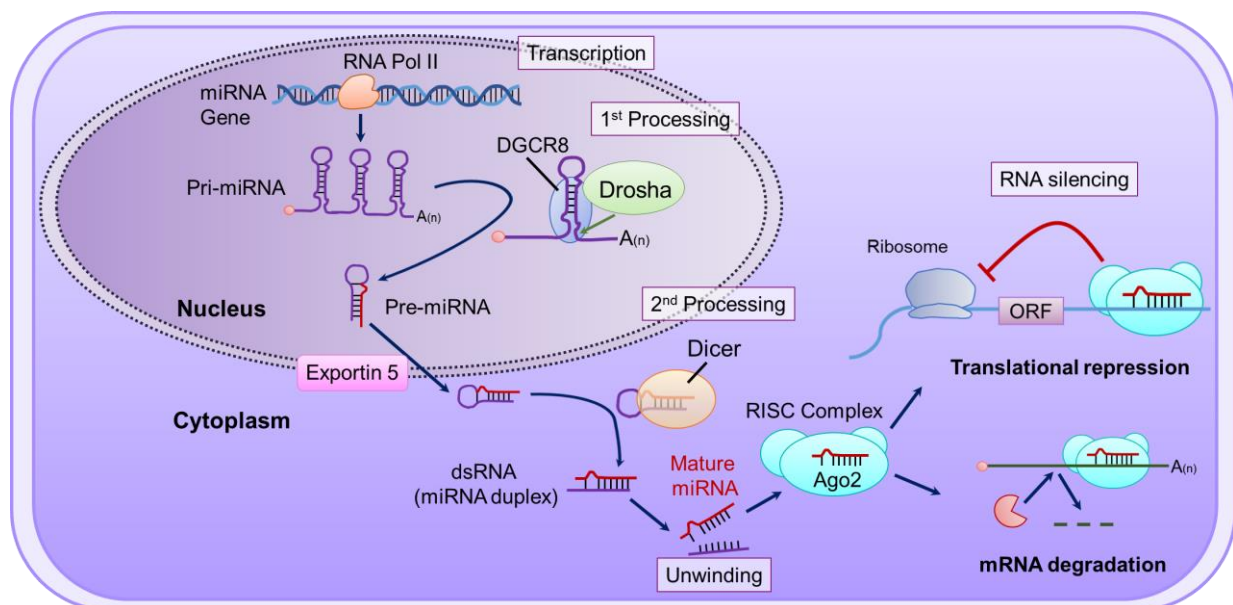


Figure 4. Canonical pathway for miRNA biogenesis and post-transcriptional regulation.

Biogenesis of miRNAs start in the nucleus, where RNA pol II mediates the transcription of a pri-miRNA. This primary transcript is processed by the protein Drosha (interacting with RNA-binding protein DGCR8) into a ~70 nucleotide precursor, pre-miRNA (1st processing). Pre-miRNAs are exported out of the nucleus and into the

cytoplasm by Exportin 5. Within the cytoplasm, pre-miRNAs are processed by Dicer into miRNA duplexes (2nd processing). These duplexes undergo a process of unwinding in which two strands are separated and only the mature miRNA strand is assembled into the RNA-induced silencing complex (RISC) by binding to Argonaute 2 protein (Ago2). The mature miRNA guides the RISC complex to target messenger RNAs (mRNAs) that have a sequence complementarity with the miRNA. This complex can mediate silencing of target mRNAs by translational repression or mRNA degradation. ORF, open reading frame. (*Diagram adapted from* (Ha and Kim 2014) *and* (He and Hannon 2004)).

In the cytoplasm, the endonuclease Dicer cleaves the loop domain of the pre-miRNA liberating the mature miRNA, a small double stranded RNA (dsRNA) molecule of ~22 nucleotides of length that is formed by both the mature miRNA strand (that will participate in the RNA silencing) and the complementary strand (which will typically be degraded) (Knight and Bass 2001). However, depending on the cellular context, both strands or primarily the complementary strand may play a role in the silencing process (Yang et al. 2011), therefore, the strand from 5' end is referred to *5p* whereas the one from the 3' end is called *3p*. The miRNA duplex undergoes a process of strand unwinding, separating the mature miRNA from the complementary strand by interaction with Argonaute2 (Ago2) (Khvorova, Reynolds, and Jayasena 2003). Afterwards, the mature miRNA is incorporated in the RNA-induced silencing complex (RISC) protein complex and guides it to target mRNAs with a complementarity to the miRNA (Kim 2005).

In addition to this canonical pathway of miRNA biogenesis involving Drosha cleavage, multiple alternative pathways have been elucidated, the majority of which use different combinations of the proteins that participate in the canonical pathway. In fact, miRNAs from introns of mRNA during splicing have been reported to mimic pre-miRNAs structure without being processed by Drosha (Ruby, Jan, and Bartel 2007). Additionally, other miRNAs have been shown to be processed by Drosha but follow a Dicer-independent pathway, although they still need Ago2 to complete their biogenesis (Yang and Lai 2010). In any case, miRNA-mediated recognition of target mRNAs promotes silencing and suppressed expression of the target genes (Hammond 2015; He and Hannon 2004).

1.4.3 Regulation of mRNA by miRNAs

MiRNAs have been shown to play important roles in the maintenance of cellular function. Their primary activity consists in regulating gene expression post-transcriptionally, by binding to the 3'-UTR of the mRNAs via the seed region, essential for target recognition and located from position 2 on the 5' end of the miRNA sequence (Hu and Bruno 2011). Furthermore, there are 2 known binding types, perfect or imperfect. Perfect Watson-Crick binding is complementary between the 5' end of the miRNA and the 3'-UTR of the mRNA, although some miRNAs bind the 5'-UTR as well; whereas the second type is an imperfect binding between the 5' end of the miRNA and the 3'-UTR region of the mRNA, although it shows a compensatory binding at the 3' end of the mRNA molecules (Brennecke et al. 2005). The miRNA-mediated target recognition either represses translation or enhances the degradation of the mRNA (Valencia-Sanchez et al. 2006). Current models of miRNA targeting also suggest that the inactivation of the target mRNA starts by repressing its translation at first and subsequently continuing with its degradation (Meijer et al. 2013). All these mechanisms have in common the destabilization of target mRNAs which is the main cause of reduced protein levels. Moreover, a single miRNA can regulate many different mRNA targets, whereas different miRNAs can bind and suppress a single mRNA target (Lewis et al. 2003). MiRNA-mediated regulation of gene expression was originally identified to be crucial in cell differentiation, embryonic development, several signalling pathways (including JAK/STAT or TGF β), muscle growth and morphogenesis, cancer, neuroinflammation and ageing (Carthew 2006; Hausser and Zavolan 2014; Hayes, Peruzzi, and Lawler 2014; Lui, Jin, and Stevenson 2015).

1.4.4 Regulation of miRNA expression and biogenesis

Transcription of miRNA genes is regulated in a similar way to protein-coding genes. In some cases, miRNA loci are located in clusters, which leads to co-transcription of a polycistronic unit, although the individual miRNAs can be regulated at post-transcription levels (Lee et al. 2002; Roush and Slack 2008). MiRNA localisation in the genome also plays a role in their transcription regulation. Indeed, intergenic miRNAs have been shown to have their own transcription regulatory elements, including promoters (Ozsolak et al. 2008). By contrast, intronic miRNAs have been reported to either share the promoter of the host gene, or to have their own promoters just like intergenic miRNAs (Monteys et al. 2010). In addition, several

studies have shown that some miRNAs may participate in regulatory feed-back loops that modulate their own expression (Spizzo et al. 2010).

Levels of miRNAs can also be regulated by modulation of post-transcriptional miRNA biogenesis and processing. Indeed, Drosha and DGCR8 influence each other's levels, and maintaining the ratio may be essential for Drosha processing activity (Han et al. 2009). Similarly, Dicer stabilisation is modulated by some of its protein partners such as TRBP, since low levels of TRBP have been shown to be concomitant with decreased Dicer expression and hindered miRNA processing (Chendrimada et al. 2005). Also, several binding proteins have been reported to regulate, both negatively and positively, miRNA processing. For instance, Lin28 has been reported to inhibit Drosha and Dicer cleavage by binding to pri- or -pre-miRNA loop region (Lightfoot et al. 2011; Viswanathan, Daley, and Gregory 2008). In contrast, p68 and p72 helicases, components of the Drosha complex, have been suggested to promote the processing of pri-miRNAs in mouse (Fukuda et al. 2007). Recent studies have also reported that alterations in the sequence of the terminal loop of a pri-miRNA may lead to rearrangements in the structure that increase miRNA processing, as observed for miR-30c in HEK cells *in vitro* (Fernandez et al. 2017). Furthermore, another species of non-coding RNA, the long non-coding RNAs (>200 nucleotides) have been reported to modulate post-transcriptional miRNA levels, mainly acting as molecular sponges (or competing endogenous RNAs), thereby binding and negatively regulating several miRNAs in different types of cancer such as osteosarcoma, prostate cancer or glioblastoma (Cai et al. 2015; Du et al. 2016; Zhou et al. 2016). Undoubtedly, regulatory factors of miRNA expression are still being investigated but some of the regulatory mechanisms described above seem to be essential for the proper transcription, processing and modulation of miRNA production.

1.5 DNA methylation and its role in gene expression

1.5.1 DNA methylation as an epigenetic mechanism

DNA methylation is well conserved among animals, plants and fungi and it was first described in the 1970s, being one of the best characterised epigenetic mechanisms to date (Bird, Taggart, and Smith 1979; Riggs 1975). In mammals, DNA methylation is primarily confined to CG dinucleotides termed CpG sites (a cytosine and a guanine separated by only one

phosphate group), which are infrequent in the genome and normally packed in forming the so-called “CpG islands” (CGIs) (Bird 1980, 2002). These CGIs are often found in association with genes and regulatory regions, mostly colocalising with promoters (Antequera 2003; Cayrou et al. 2011; Larsen et al. 1992). CGIs have been considered to be mostly unmethylated, while the rest of the genome is considered to be methylated (Bird et al. 1985; Deaton and Bird 2011; Wang et al. 1997). Nonetheless, some studies using genome-wide profiling of DNA methylation have supported that CpG islands may also be predominantly methylated in some cases, especially those inside genes (Jones 2012; Shen et al. 2007). Methylation of CGIs has been observed in normal tissues promoting genomic imprinting, X-inactivation or cell specification between tissues, as well as in malignant cells inducing inappropriate silencing (Illingworth and Bird 2009). However, deregulated demethylation of certain promoters has also been reported in pathological conditions (e.g. PD, cancer) (Matsumoto et al. 2010; Yanagawa et al. 2004). These observations suggest a fine balance between methylation and demethylation of promoters that appears to be essential for proper cellular function. The mechanisms underlying these changing patterns of methylation in CGIs still need further research, in order to shed light on their contribution to physiological processes and pathologies (Jeziorska et al. 2017).

During DNA methylation, there is covalent transfer of a methyl group from S-adenosyl methionine to the carbon in position five of the cytosine ring, producing 5-methylcytosine (5mC) (Hussain et al. 2013). This modification is generally associated with repressed chromatin and inhibition of gene expression, since methylation has been implicated in changing protein-DNA interactions, inhibiting association of important DNA-binding factors and promoting modifications in chromatin structure (Jones et al. 1998; Yoon et al. 2003). These methyl-CpG-binding proteins interact with co-repressor factors (e.g. histone deacetylases) that play a role in modulating transcription as well as chromatin remodelling and modification (Bird 2002; Klose and Bird 2006; Smith and Meissner 2013).

1.5.2 Regulation of DNA methylation

DNA methylation is a reversible process that contributes to the regulation of gene expression throughout the life of the organism. The methylation pattern is mediated by the DNA methyltransferase (DNMT) family, which is well conserved and comprises DNMT1, DNMT3A and DNMT3B, considered the canonical DNMTs in mammals (**Figure 5**) (Laisné

et al. 2018). The maintenance of DNA methylation is mostly performed by DNMT1, whereas DNMT3A and B carry out *de novo* methylation in previously unmethylated CGIs (Okano et al. 1999; Song et al. 2011). As opposed to DNMTs and methylation, DNA demethylation is often carried out by proteins of the ten-eleven translocation (TET) family (Williams, Christensen, and Helin 2012). Effects of methylation on the genome also depend on the interaction of DNMTs with transcription factors, DNA-binding proteins and non-coding RNAs (e.g. miRNAs) in addition to histone marks and sequence modifiers (Jones 2012; Lyko 2018). Genomic DNA methylation in mammals is suggested to represent cell-intrinsic regulation that is encoded in the DNA sequence by CG density or binding regions for transcription factors, thus being an essential mark in the contribution of cell fate determination (Schübeler 2015; Yadav, Quivy, and Almouzni 2018).

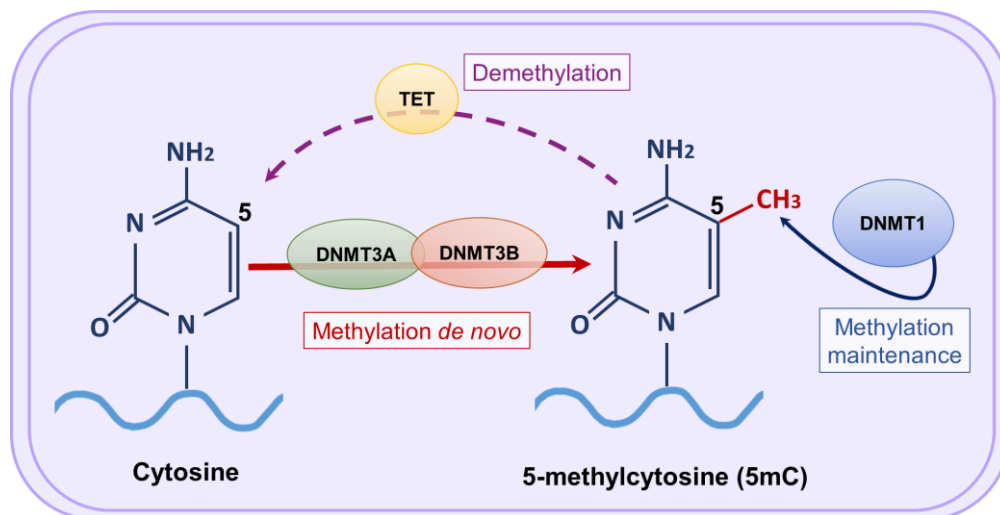


Figure 5. Role of DNMTs in DNA methylation.

DNA methylation is characterised by the addition of a methyl group onto the fifth position of a cytosine ring resulting in 5mC. The establishment of the methylation pattern *de novo* is mediated by DNMT3A and 3B. The maintenance of this methylated state is performed by DNMT1. This modification is generally related to repressed chromatin and inhibition of gene expression. Proteins from the TET family show an opposite activity to DNMTs and mediate demethylation of the DNA, from 5mC to demethylated cytosine. (Diagram by Eduardo Frías Anaya).

1.6 MiRNAs and DNA methylation in the ageing BBB

1.6.1 *The role of miRNAs in the ageing BBB*

Genetics and epigenetics are suggested to accelerate or delay the onset of ageing and age-related diseases. As established above, miRNAs have a role in regulating different genes at once, therefore contributing to the modulation of several interlinked pathways, including inflammation, vascular diseases or ageing, in human and different animal models and in different organs including the brain (Jazbutyte et al. 2013; O'Connell et al. 2010; Sun et al. 2012, 2013). Accordingly, recent studies have proposed miRNAs as potential sensors of ageing and cellular senescence, contributing to the complexity of the age-related changes (Harries 2014; Schroen and Heymans 2012).

MiRNAs have also been implicated in ageing of the BBB. Indeed, different miRNAs have been recently found to be expressed in the BBB, targeting genes related to BBB permeability. For instance, miR-132 has been reported to attenuate brain injury in a mouse model of stroke by repressing MMP9 activity (Zuo et al. 2019). By contrast, MiR-212/132 expression induced by hypoxia in mouse and human BECs promotes downregulation of junctional protein such as Cldn1 or Jam3 (Burek et al. 2019). Similarly, miR-155 has been suggested to be a negative modulator of BBB function in neuroinflammation, by repressing TJ (e.g. Cldn1) and focal adhesion proteins (e.g. DOCK1) in both the mouse model of experimental allergic encephalomyelitis (EAE) *in vivo* and cultured human BECs *in vitro* (Lopez-Ramirez et al. 2014). These findings suggest that miRNAs participate in BBB integrity modulation. In addition, recent studies have also reported age-related changes in several miRNAs in the mouse and human BBB *ex vivo*, mostly associated with deregulation of DNA-binding genes related to stress and immune response as well as apoptosis/autophagy pathways (Goodall et al. 2019). Indeed, the same study has reported age-related overexpression of miR-155, which may be playing a similar role as the one observed in neuroinflammation, but also downregulation of miRNAs such as miR-182 and 183, which promote neuroprotection in conditions of oxygen and glucose deprivation (Bernstock et al. 2016). Taken together these observations suggest a potential role of miRNA deregulation in cerebrovascular dysfunction during ageing. However, future studies are needed in order to better understand the underlying mechanisms and how they actually impact on the age-mediated BBB disruption.

1.6.2 DNA methylation in the ageing BBB

DNA methylation processes are also altered during ageing. Overall, ageing promotes an interesting pattern, with a global genomic hypomethylation observed in several species, including human and rodents, and in different organs including the brain, while specific CGIs may get hypermethylated, thereby promoting a mosaic-like distribution of methylation patterns in the aged genome (Bollati et al. 2009; Hannum et al. 2013). In addition, DNMT activity appears to be altered during ageing, including downregulation or upregulation patterns of the three canonical DNMTs, DNMT1, 3A and 3B, which add to the complexity of the ageing process (Lopatina et al. 2002; Oliveira, Hemstedt, and Bading 2012; Xiao et al. 2008). Aberrant DNA methylation patterns have also been reported in the ageing brain, including region-specific alterations in genes that are involved in CNS development, neuron differentiation and neurogenesis (Davies et al. 2012; Horvath et al. 2012). DNA methylation changes also appear to have an effect on the integrity of the BBB, although its potential effect in the ageing BBB is largely unknown. Indeed, DNA methylation changes have been implicated in BBB permeability by modulating MMP activity in mice after stroke (Mondal et al. 2019). Also, although not in the context of ageing, previous studies have reported that miRNA-mediated downregulation of DNMT3B promotes increased BBB permeability, by indirect overexpression of MMP9 in cultured mouse BECs exposed to hyperhomocysteinemia (Kalani et al. 2014). Additionally, even though it has not been described in the BBB, DNA methylation patterns have been reported to be important for vascular function and pathogenesis in animal models (Nguyen et al. 2016; Toghiani et al. 2015). Taken together, these observations suggest that DNA methylation plays a role in ageing and vascular function, and it may contribute to BBB function. Nonetheless, further research is needed to determine if the age-related changes observed in methylation pattern have an impact on cerebrovascular integrity.

1.7 Chronic inflammation contributing to ageing: Inflammageing

1.7.1 Inflammageing: definition and causes

Inflammageing is the name given to the contribution of a chronic inflammation process to ageing in the absence of overt infection, characterised by high levels of pro-inflammatory molecules in cells and tissues, and it was first described by Franceschi and colleagues

(Franceschi et al. 2000). The theory of inflammageing considers that due to continuous antigenic load and stress throughout life, inflammatory response becomes detrimental during ageing and is characterized by a counterbalance between pro- and anti-inflammatory cytokines that ends up promoting a pro-inflammatory state (Lio et al. 2003; De Martinis et al. 2005). Moreover, as proposed by Franceschi and colleagues, there is a combination of a biological inflammatory background, induced by exposure to inflammatory stimuli over time, and the presence of frail or absence of robust gene variants that would be expressed in specific cells and organs, which would explain why some individuals are more susceptible to certain age-related diseases with inflammatory components (Pericak-Vance and Haines 1995; Shulman et al. 2013). Accordingly, the immune system has been reported to promote an age-related increase of inflammatory cytokine circulating levels, including interleukin-1 (IL-1), interleukin-6 (IL-6) or TNF- α , which suggest a contribution to chronic inflammation intensified with ageing (Bruunsgaard 2006; Sergio 2008). In addition, the inflammasome, a multiprotein complex implicated in activating inflammatory signalling pathways and inducing secretion of several cytokines including IL-1 β (Rathinam, Vanaja, and Fitzgerald 2012), has been shown to participate in inflammageing and to promote detrimental effects in several aged tissues, including the brain (Mejias et al. 2018). These pro-inflammatory processes induce significant overexpression of genes related to oxidative stress and inflammation, as well as exacerbating the immune response (C. K. Lee, Weindruch, and Prolla 2000). These alterations in expression participate in perpetuating a feed-forward cycle that contributes to the chronic inflammation observed in ageing, a low-grade and persistent phenomenon that induces responses leading to tissue degeneration (Ferrucci and Fabbri 2018).

The mechanisms that contribute to inflammageing appear to be numerous and highly related to the immune state of the organism. One of the possible sources of inflammageing may be immunosenescence, which is the age-related decline in the immune system activity due to a lifelong exposure to pathogens, immune cells modifications/activation and genetic predispositions (McElhaney and Effros 2009; Shaw et al. 2010). Similarly, senescent cells normally express a senescence associated secretory phenotype (SASP) that results in secretion of cytokines, exacerbating the pro-inflammatory environment and the decline of normal tissue function (Coppé et al. 2010). The presence of circulating pro-inflammatory miRNAs (e.g. miR-21, miR-126 or miR-146a) in blood may also contribute to the activation of immune response and vascular function in ageing (Olivieri, Lazzarini, et al. 2013; Olivieri, Rippo, et al. 2013). Age-related mitochondrial dysfunction has also been described to contribute to inflammation

by ROS production and cytokine secretion (Lerner, Sundar, and Rahman 2016). In addition to all of the inflammatory factors described above, some studies have proposed the endogenous cell debris to be an important stimulus in inflammageing, which suggests an auto-inflammatory component to inflammageing (Franceschi et al. 2016).

1.7.2 Inflammageing effect on the BBB

CNS ageing is also associated with a chronic low-grade inflammation that influences the function of all brain cell types, including the cells of the BBB (Grammas 2011; Stichel and Luebbert 2007). Despite the fact that inflammatory processes occurring on the ageing BBB are often related to neurodegeneration and neurological diseases exclusively (Takeda et al. 2013; Zlokovic 2005), several studies have assessed the effect of the age-related inflammatory state on neurovascular dysfunction during healthy ageing. In fact, BEC activation and cytokine secretion have been reported to promote increased BBB permeability and recruitment of immune cells under challenging conditions in aged mice and non-human primates (Reyes, Fabry, and Coe 1999; Wei et al. 2000). Accordingly, increase in circulating pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-6) has been reported to increase expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in BECs (Trickler, Mayhan, and Miller 2005), which is an essential transcription factor in inflammatory responses and induces secretion of TNF- α and IL-1 β (Blackwell and Christman 1997; Hayden and Ghosh 2014). Also, astrocytes have been reported to express SASP during ageing, thereby secreting pro-inflammatory modulators that induce cerebrovascular inflammation (Salminen et al. 2011). However, there exists some controversy regarding BBB dysfunction in normal, healthy ageing. Indeed, brain microvessels have been reported to show decreased expression of cytokines with ageing, including TNF- α and IL-1 β , in rats (Tripathy et al. 2010), whereas BBB breakdown has been associated with inflammatory processes in the ageing brain in humans, including upregulation of cytokines and chemokines (e.g. IL-8) (Bowman et al. 2018). In addition, more recent reports have described that the healthy aged BBB is characterized by a significant inflammatory process and a concomitant disruption of TJ proteins, but not by recruitment of immune cells into the brain (Elahy et al. 2015). Taken together, these findings suggest that the process of inflammageing affects the cerebrovasculature at several levels in a precise way. Nevertheless, the underlying mechanisms related to healthy ageing in absence of pathology need further analysis in order to assess their real impact on BBB function and age-related disruption.

1.8 Sex differences in the ageing blood-brain barrier

Despite most of the available literature has been obtained studying the male cerebrovascular, the potential role of sex in certain processes must be considered. Indeed, sex differences have been observed in mitochondrial fission (division) and fusion (merging), which modulate the organelle shape, in mouse cortical astrocytes, suggesting potential effects on mitochondrial function and energy balance (Arnold, de Araújo, and Beyer 2008; Arnold, Victor, and Beyer 2012). Microglial cells have been reported to exhibit sexual dimorphism, with female mouse microglia exerting a neuroprotective role after ischemic stroke when transplanted into a male mouse brain previously lacking endogenous microglia (Lenz and McCarthy 2015; Villa et al. 2018). Also, DNMTs, and specifically DNMT3A, have been reported to be differentially expressed between male and female rats in certain brain regions, with higher levels of DNMT3A in the female amygdala during brain development (Kolodkin and Auger 2011). Similarly, sex differences in longevity and ageing patterns have been observed in most sexually reproducing species, with females usually living longer than males (Regan and Partridge 2013). Higher life expectancy for women can be linked to greater incidence of certain neurodegenerative disorders such as AD (Altmann et al. 2014; Paganini-hill and Henderson 1994), as well as to higher tendency to suffer cerebrovascular incidents and stroke. For instance, although women are generally more protected against neurovascular dysfunction than men over the life course, post-menopausal women have been reported to be at higher risk to suffer neurovascular incidents (e.g. stroke), and have less favourable outcomes than men of the same age (Chisholm and Sohrabji 2016; Reeves et al. 2008). The higher incidence of stroke, in addition to the fact that Alzheimer's and other neurodegenerative diseases have a vascular component (Desai et al. 2007; Rosenberg 2014; Weller, Boche, and Nicoll 2009), suggest that sex-dependent differences during ageing may also involve changes in cerebrovasculature.

Some of these differential processes appear to be associated with the role of sex hormones, whose levels have been shown to drastically change during reproductive senescence (Maffucci and Gore 2006). Interestingly, apart from their reproductive role, sex steroid hormones have been reported to participate in brain function (Moraga-Amaro et al. 2018). In fact, oestrogen and progesterone contribute to neuroprotection in several conditions including

neuroinflammation, oxidative stress and ischemic injury in both women and female rodents (Garay et al. 2007; Gavin et al. 2009). Specifically, oestrogen has been described to exert anti-inflammatory effects on cerebrovascular endothelial cells and BBB, by blocking NF- κ B signalling and cytokine production or reducing expression of adhesion molecules that mediate leukocyte recruitment across the brain endothelium (Galea et al. 2002; Mori et al. 2004). Oestrogen also shows beneficial effects on BECs by increasing mitochondrial efficiency and decreasing free ROS production, thereby promoting both cell survival and angiogenesis (Zeevi et al. 2010). Similarly, progesterone has been implicated in attenuating TJ degradation in rats by reducing MMP activity following ischemic injury (Ishrat et al. 2010). Additionally, testosterone is also present in the female brain despite being predominantly a male hormone. Testosterone can be metabolized into estradiol (more common form of circulating oestrogen) by aromatase or into a more potent androgen by 5- α reductase, and the balance of androgens/oestrogens might determine the final hormonal effect in endothelial function, oxidative stress and inflammatory responses in cerebral vessels (Krause, Duckles, and Gonzales 2011; Miller and Duckles 2008). These observations suggest that sex hormones, and mostly oestrogen and progesterone, mediate modulation of the cerebrovascular function, whereas the potential role of testosterone in the BBB function requires further research.

Thus, the age-dependent reduction in hormone levels with reproductive senescence or menopause, might have some severe effects on brain and BBB function. Accordingly, previous studies suggest that menopausal depletion in sex steroid hormones is a risk factor in ageing and age-related diseases, showing an effect in brain function and BBB properties (Lutescu et al. 2007; Siddiqui et al. 2016). In fact, progesterone neuroprotective role is decreased in the ageing female brain, although it appears to be recovered in post-menopausal hormone therapies (Tanaka et al. 2018). By contrast, oestrogen protective effect has been proved to become attenuated following reproductive senescence (Maggioli et al. 2016; Sunday et al. 2007), however, the administration of oestrogen as part of hormonal therapies has been shown to worsen neural impairment due to neuroinflammation in aged female rodents, while increasing the risk of developing stroke in post-menopausal women (Marriott et al. 2002; Wassertheil-Smoller et al. 2003). Strikingly, oestrogen has been shown to differentially affect the cerebrovascular function and the BBB integrity depending on the age in female rodents (Bake and Sohrabji 2004), which is consistent with a worsen BBB integrity in aged females compared to young females and also age-matched males. These observations are suggesting potential

changes at the molecular level that promote the shift in oestrogen protection towards a detrimental effect, although the real alterations are largely unknown.

Therefore, the study of both, the mechanisms responsible for age-related BBB dysfunction and the sex differences observed in ageing, might aid understanding the potential synergy between them and the real impact on the female cerebrovasculature.

1.9 Hypothesis and objectives of the present study

BBB dysfunction, both functionally and structurally, is a common phenomenon in the ageing CNS. Modifications in the gene expression profile mediated by epigenetic mechanisms, such as miRNAs, are thought to modulate BBB breakdown (Tominaga et al. 2015). Deregulation of miRNAs has been reported in the ageing brain and BBB in several species (Goodall et al. 2019; Inukai et al. 2012), however, the molecular mechanisms underlying miRNA-mediated gene expression regulation and its role in BBB dysfunction during healthy ageing are not completely understood. Moreover, most of the knowledge available in this field has been obtained studying male physiology, despite the fact that post-menopausal women show a higher tendency to develop neurovascular incidents (Towfighi et al. 2007). Thus, a better understanding of structural and molecular age-related alterations in the female BBB is required in order to assess the functional changes and sex differences observed in cerebrovascular ageing.

This PhD project has investigated the hypothesis that functional and structural alterations observed at the ageing female BBB are related to changes in gene expression and miRNA deregulation.

Hence, the aims of the present study were:

- 1) To analyse ultrastructural alterations in the ageing female mouse BBB using transmission electron microscopy (TEM).
- 2) To investigate the molecular features that underlie ageing in the BBB of female mice, using RNA sequencing analysis.

- 3) To determine the putative role of selected age-deregulated pairs of miRNA and target mRNA in age-related changes in BBB function.

- 4) To validate the actions of selected miRNA/mRNA pair on BBB permeability and leukocyte adhesion *in vitro*.

Chapter 2. Ultrastructural alterations in the ageing female blood brain barrier.

2.1 Introduction

The blood-brain barrier (BBB) is essential for the correct function of central nervous system, as well as to protect the brain parenchyma and maintain its homeostatic balance. The BBB is known to experience a breakdown during physiological ageing in different species including mouse, rat and human. Age-related changes to the BBB have been described at functional, cellular and molecular levels. Functional changes include BBB breakdown and increased permeability in several brain regions such as hippocampus and cortex (Cullen, Kócsi, and Stone 2005; Montagne et al. 2015; Simpson et al. 2010). Although the vast majority of studies have been performed in males, age-related BBB alterations have also been described in females, for instance, Elahy and colleagues have shown that the BBB is impaired in aged female mice and shows higher capillary permeability, attenuation of tight junction proteins, occludin-1 and ZO-1, and increased inflammation by TNF- α overexpression in BECs (Elahy et al. 2015). Also, earlier studies by Bake and colleagues showed an age-dependent decrease in the expression of the tight junction protein Cldn-5 in older, reproductively senescent female rats when compared to younger females (Bake and Sohrabji 2004). Interestingly, there appear to be sex differences regarding age-related alterations of the BBB. Indeed, Bake and colleagues also reported that age-dependent BBB disruption is paralleled by Cldn-5 mislocalisation in ageing female rats but not in age-matched males (Bake, Friedman, and Sohrabji 2009). The same study showed a similar disruption in microvessel Cldn-5 in post-menopausal women compared to young women, which suggests that something similar could be happening in the human brain, even though they did not analyse men brains. Also, microvessels from oestrogen-sensitive brainstem regions in ageing female hamsters have been shown to present age-related changes such as TJ disruption and degenerative inclusions in pericytes (Gerrits et al. 2010). These findings suggest sex-dependent alterations in BBB dysfunction with ageing that might also be present at the ultrastructural level.

Specific structural changes have also been observed in almost every cellular and subcellular component of the BBB, although as mentioned above these alterations have mainly been assessed in males. For example, according to early studies, the number of BECs appears to decrease in human and primate BBB with ageing, although a similar reduction has not been

observed in rodents (Burns, Kruckeberg, and Gaetano 1981; Mooradian 1988). Similarly, BEC luminal surface was reported to become irregular during ageing, with higher presence of pseudopod-like protrusions pinching off of the endothelial cytoplasm into the lumen (E. Y. Lee et al. 2000). The number of endothelial mitochondria in both the cortex and hippocampus has also been shown to decrease in the primate, mouse, rat and human brain (Hicks et al. 1983). In addition, one of the most characteristic structural changes observed during ageing is the increase in thickness of the BM, which can double in the microvessels of aged mice compared to young mice (Ceafalan et al. 2019). This morphological change is accompanied by biochemical alterations including changed levels of extracellular proteins such as collagen IV, laminin and other proteoglycans (Candiello et al. 2010).

Pericytes and astrocytes also show alterations during the process of ageing. A decrease in pericyte numbers in human cerebral white matter (but not grey matter) has been previously reported in aged male mice (Farrell et al. 1987). However, number of pericytes appears to be increased in male rat cortex (Peinado et al. 1998), whereas no change is found in monkeys (Peters, Josephson, and Vincent 1991). In addition, previous publications have described loss of coverage and contact between aged pericytes and BECs (Bell et al. 2010; Hughes et al. 2006) and a decline in pericyte area/vessel profile in human BBB (P. A. Stewart et al. 1987). It has also been shown that mitochondrial area in pericytes is increased during ageing in both cortex and hippocampus of rats (Hicks et al. 1983; Mooradian 1988). During age-related astrogliosis, astrocytes show morphological changes into a swollen phenotype with thicker projections, accumulating in the ageing cortex, hippocampus and other brain regions (Amenta et al. 1998; Kanaan, Kordower, and Collier 2010); however, astrocyte number does not appear to change in the ageing brain, at least in humans (Fabricius, Jacobsen, and Pakkenberg 2013). Aged astrocytes also express a marked deregulation at the levels of cytoplasmic antigens, membrane proteins and growth factors (Cotrina and Nedergaard 2002) and, when activated, have a secretory phenotype which includes production of cytokines and neurotrophic factors (Clarke et al. 2018). In addition to this senescent, pro-inflammatory phenotype, ageing has been recently reported to induce neurovascular dysfunction by reducing astrocytic end-feet contacts on brain vessels (Duncombe et al. 2017). This pro-inflammatory environment in the ageing brain might link back to ultrastructural changes on BECs, which have been reported to form the beforementioned pseudopods also in neuroinflammatory conditions and as a response to cerebral ischemia (Øynebråten et al. 2015; Pluta et al. 1991).

Because of the small size and complex interrelationship between components of the BBB, ultrastructural analysis is one of the most useful techniques to study age-related changes in the cerebral vasculature. In particular, transmission electron microscopy (TEM) is one of the most commonly used techniques to study the ultrastructure of the BBB in multiple species across different ages (Castejón 2011; Cipolla et al. 2004; Haley and Lawrence 2017; Hawkes et al. 2013; Hirano, Kawanami, and Llena 1994; Nahirney, Reeson, and Brown 2016). Normally, TEM involves collecting serial tissue sections which are then imaged at high resolution. These 2D images can be used to analyse structure, number or interactions between cellular and subcellular elements of the BBB. However, this technique requires a considerable amount of manual labour when analysing several sections. In addition, a certain number of factors, such as section folding during the placement or electron beam damage, can lead to discontinuous or interrupted series as well as imperfect image stacks and therefore introduce artefacts which may affect interpretation (Knott et al. 2008). Automation protocols have significantly improved the efficiency of TEM image acquisition and processing, including image stacking and montage compilation, which has enabled the development of 3D reconstruction TEM techniques. This approach has several benefits over 2D analysis, such as in-depth analysis of structures of interest including volume, localisation in space and intimate contact between BBB elements alongside a whole reconstructed vessel (Mathiisen et al. 2010). However, despite the deeper insight allowed by 3D reconstruction analysis, it does have several limitations such as being restricted to particular structures, being highly time consuming and depending on the quality and size of the image stack for a successful outcome.

Sex differences are undoubtedly multifaceted, and one of the common explanations for such differences, including those at the cerebrovascular level, relies on the role of sex steroid hormones such as oestrogen and progesterone, whose levels drastically change with menopause and reproductive senescence (Maffucci and Gore 2006; Sherman, West, and Korenman 1976). Experiments on stroke models of middle cerebral artery occlusion have shown smaller effects in young adult female rats and mice when compared to males, a protection that decreases when female rodents are ovariectomised and sex steroid hormone levels are altered (Park et al. 2006; Selvamani and Sohrabji 2010). Precisely, oestrogen and progesterone have been described to target brain vessels and intervene in regulation of BBB permeability and function in women and female mice (Krause, Duckles, and Pelligrino 2006; Wilson et al. 2008). During reproductive senescence, oestrogen decline has been reported to promote loss of BBB integrity and altered transport within the female mouse brain (Kastin, Akerstrom, and Maness 2001;

Wilson et al. 2008), as well as to hinder amyloid- β clearance in the human cortical cerebrovasculature (Li et al. 2000). Similarly, oestrogen-mediated anti-inflammatory effects also get compromised during ageing in female mice (Maggioli et al. 2016). These observations suggest that age-dependent changes in sex steroid hormones contribute to BBB alterations, with reproductive senescence having a greater impact on the BBB of females over males.

Although much is known about the BBB in ageing, ultrastructural changes still require thorough investigation. Also, despite the evidence of sex differences across age, the vast majority of studies looking at age-related BBB dysfunction have focused on males, and there is still a lack of information regarding how the female BBB is structurally and functionally altered during ageing. Therefore, the aim of this chapter was to analyse the ultrastructural changes in cortical and hippocampal capillaries from young and aged C57BL/6J female mice. To first confirm whether our animal model replicated findings obtained in the ageing male BBB in literature, preliminary analyses of BM thickness, mitochondrial number and size and cell-cell contacts were carried out in 2D images of cortical capillaries only. However, as mentioned above, among the brain regions altered in ageing, two of the most understood and studied are cortex and hippocampus, which undergo processes of age-related shrinking, inflammation and BBB dysfunction (Popescu et al. 2009; Viggars et al. 2011). For that reason, when performing the more in-depth 3D reconstruction analysis, hippocampal capillaries were analysed together with cortical capillaries in order to assess potential differences between these regions.

2.2 Material and Methods

2.2.1 Animals

6 and 24-month-old female C57BL/6J mice were used in this study. All animal work was approved by The Open University Animal Welfare and Ethics Research Board and the UK Home Office (PPL 80/2612). Animals were housed on a 12-hour light/dark cycle. Food and water were provided *ad libitum*.

2.2.2 Oestrous cycle monitoring

To determine the stage of oestrous cycle, the protocol published by Byers and colleagues was followed (Byers et al. 2012). Vaginal lavage (approximately 0.2-0.4 ml of distilled water) was blindly carried out on young and aged mice at the start of the light phase for 7 consecutive days. The vaginal smears were examined under an LCD Brightfield Microscope (Bresser, Rhede, Germany). The stage of the oestrous cycle was classified as i) pro-oestrous if there were numerous nucleated epithelial cells and some cornified cells, ii) oestrous if there were cornified cells with some nucleated and non-nucleated epithelial cells, iii) metoestrous if there were numerous cells including cornified and non-nucleated, leukocytes start appearing and iv) dioestrous if there were mainly leukocytes and occasionally some non-nucleated cells.

2.2.2.1 Measurement of reproductive hormones in plasma of young and aged female mice

Blood was collected from anaesthetised young and aged female mice (n=6/group). Plasma was separated by centrifuging (2000g, 3min) and stored at -80 °C. Concentrations of progesterone (#80559, Crystal Chem., Zaandam, Netherlands) and estradiol (#ES180S-100, Calbiotech, CA, USA), the most active type of oestrogen, were measured using ELISA kits according to manufacturer's instructions. The ratio of progesterone/estradiol (P/E2) was calculated for both age groups.

2.2.3 Brain collection and processing for TEM

2.2.3.1 Perfusion and brain collection

6 and 24-month-old female C57BL/6J female mice were used in this study. Mice were intraperitoneally injected with an overdose of pentobarbitine sodium (20% w/v; Pentject, Animalcare, UK) and perfused intracardially with 0.01M phosphate-buffered saline (PBS, 0.1M phosphate buffer (PB, Na₂HPO₄ 10mM, KH₂PO₄ 1.8 mM, 0.1% Tween) and 0.9% sodium chloride; Sigma-Aldrich, Dorset, UK). After PBS, mice were perfused with 3% paraformaldehyde (PFA, Sigma-Aldrich, Dorset, UK) and 1% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1 M PB (pH 7.4). Brains were then extracted and maintained in 2.5% glutaraldehyde in 0.1 M PB for 5 days.

After the post-fixation time, brains were sliced coronally using a vibratome into slices of 50 μm thickness. Slices containing either prefrontal cortex or dorsal hippocampus were selected to be used for EM and stored in 0.1 M PB at 4 °C prior further processing.

2.2.3.2 *Slice processing*

Brain slices were post-fixed in 2.5% glutaraldehyde in PB 0.1M for 1h at RT and then rinsed 3 times for 10 min each in 0.1M PB. Afterwards, slices were incubated in 1% osmium tetroxide (OsO_4 , Agar Scientific, Essex, UK) in PB 0.1M for 1 hour at RT. Tissues were dehydrated by passing through increasing graded aqueous solutions of ethanol from 30% to 100% (each for 10 min). After 100% ethanol, slices were incubated in 100% acetone for 3 times (10 min each). Then samples were impregnated with a mixture of 50% epoxy resin (Epon 812 epoxy resin, #45345, Sigma-Aldrich, Dorset, UK) and 50% pure acetone (50:50) for 2h at RT. The following steps were performed with the help of Radka Gromnicova and Igor Kraev.

2.2.3.3 *Resin Embedding*

Using a paintbrush, a small amount of 50:50 resin:acetone mixture was placed on a cleaned piece of aclar plastic film. Slices were then transferred on top of the film and air-dried to allow acetone evaporation. Then, a drop of pure resin was placed on them quickly and gently and excess resin was eliminated using filter paper. Another film of aclar was then slowly placed on top, ensuring all the bubbles were removed. Sections between both films of aclar were placed inside the oven and incubated at 60 °C. After the first 15 min, weights were placed on top to make sure excess resin were removed. Samples were left for 48h to ensure polymerisation of the resin.

2.2.3.4 *Capsule preparation*

Gelatine capsules were filled with an identifying number. A drop of resin was also pipetted inside each capsule. Capsules were then placed upside down on rubber platforms and incubated in the oven at 60 °C for 48h to polymerise. The aclar sheets were peeled off one from the other, trying to keep all the slices stuck in one of the sheets. A drop of pure resin was pipetted onto each polymerised slice. A labelled capsule of polymerised resin was then placed upside-down on the resin drop and polymerised again at 60 °C for 48h. Blocks were coded, and

all further analyses were carried out with the investigator blind to the experimental status of the tissue.

2.2.3.5 *Microsectioning*

Slices embedded on the block surface were trimmed with a glass knife along the entire area of interest. A pyramid of size 30 μm x 300 μm was prepared on the face of the block. The pyramid was then serially sectioned into sections of 50 nm of thickness by a diamond knife (Diatome, Nidau, Switzerland) which includes a small metal trough that can be filled with water. Ribbons of tissue were floated on the water surface and then collected onto copper grids covered by a carbon coated pioloform film. Sections on the grids were left air drying for at least 2h. Radka Gromnicova performed the microsectioning.

2.2.3.6 *Counter-staining*

Sections were pre-treated with 1% HCl for 1 min in order to reduce formation of crystals on the tissue. After this first treatment, sections were washed 3 times in distilled water. Sections were then stained with uranyl acetate (Agar Scientific, Essex, UK) for 20 min or with Uranyless (Delta Microscopies, Mauressac, France) for 10 min and then washed 3 times in distilled water. Grids were left to air dry for 2h before imaging. Finally, the grids were stained with Reynold's lead citrate (Delta Microscopies, Mauressac, France) for 7 min, which recipe dates back to 1963 (Reynolds 1963), and washed again 3 times in distilled water. Radka Gromnicova performed the counter-staining.

2.2.3.7 *Image collection*

Series of sections were imaged on a JEM 1400 (Jeol, Tokyo, Japan) electron microscope. Images were acquired automatically using an AMT XR60 camera and SerialEM software in montaging mode, following the method described by Mastronarde (Mastronarde 2005). Acquisition was performed using a column magnification of 6000x and acceleration voltage of 80 kV. Each section was imaged taking images of 3x4 (x:y axis).

2.2.3.8 *Image 2D analysis*

TEM images for the 2D analysis were processed by Image J (<https://imagej.net/Welcome>) to trace structures of interest and obtain their areas in 50 cortical capillaries per animal. A mean value of the measures in those 50 vessels was calculated for each animal. A total of 3 animals per age group (young and aged) were used in this experiment (n=3/ age group).

2.2.3.9 *Image 3D analysis of prefrontal cortex and hippocampus*

In the case of the 3D analysis, images were first corrected for chromatic aberrations using the ImageJ plugin bUwarpJ (Arganda-carreras et al. 2006). Individual images were then stitched together into montages using Photomerge in Adobe Photoshop CS6 (Adobe). A custom script was created by the head of the TEM unit, Igor Kraev, and used in Adobe ExtendScript Toolkit CS6 in order to automate the process for batch processing. Montages were automatically aligned using the ImageJ TrakEM2 plugin (Cardona et al. 2012). After montages were properly aligned, reconstruction of capillaries was performed using Reconstruct software (<http://synapseweb.clm.utexas.edu/software-0>).

The final 3D reconstructions were smoothed, stylised and coloured using 3D Max software (Autodesk). Structures of interest were manually traced using Reconstruct in series of montages from 5 different capillaries per animal, a mean value of the measures in those 5 vessels was calculated for each animal. A total of 3 animals per age group (young and aged) were used in this experiment (n=3/ age group). Main steps of the 3D reconstruction process are summarised below (**Figure 6**). A video of a whole 3D reconstructed capillary has been added as a non-book element to this thesis (ORDO DOI: <https://doi.org/10.21954/ou.rd.12594641>).

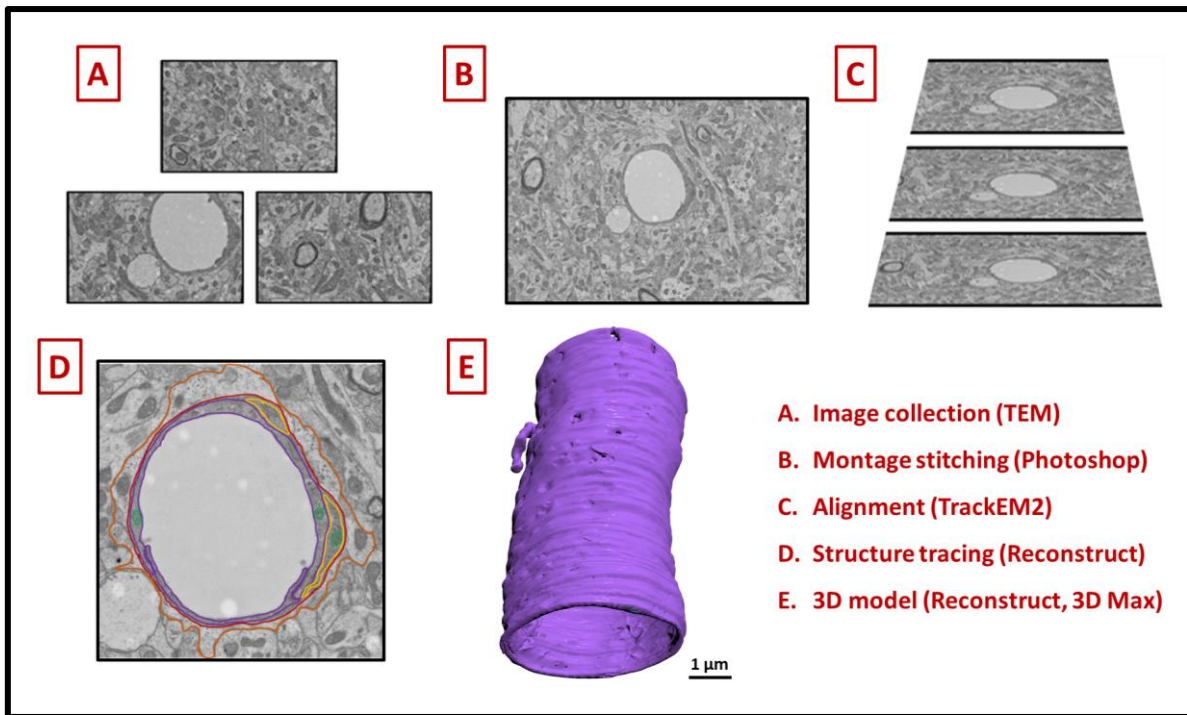


Figure 6. Protocol to obtain 3D reconstructions of images of mouse brain capillaries captured using TEM.

A) Multiple 2D images of the area of interest were collected. B) Montages were stitched together using Photoshop. C) Montages were then aligned using TrackEM2 plug in of Image J. D) Once aligned, cellular and subcellular components of interest were traced using Reconstruct software. E) At the end, same Reconstruct software was used to obtain a 3D model of the capillary. 3D Max software was used to smoothen and colour the 3D models of each cellular and subcellular component of the BBB.

Several features were measured. Volume and area of cellular and subcellular components were obtained directly from Reconstruct software. These values were used to calculate component volumes with respect to total cell volumes or contact between cells. In addition, BM thickness was obtained by 4 measures per section, and as an average value per vessel. TJ tortuosity refers to how much the junctional contact between BECs varies along the longitudinal axis of the vessel, and it was measured along the whole capillary length by taking reference points at the lumen, middle and basement membrane parts of the TJ and obtaining a mean value. In addition, TJ length or complexity was measured on the transversal section of the capillaries as the product of dividing the TJ length by the diagonal of a rectangle containing the length and height of the complete TJ (**Figure 7**) (Jackman et al. 2013). Both of these features give different information of the TJs but may be correlated.

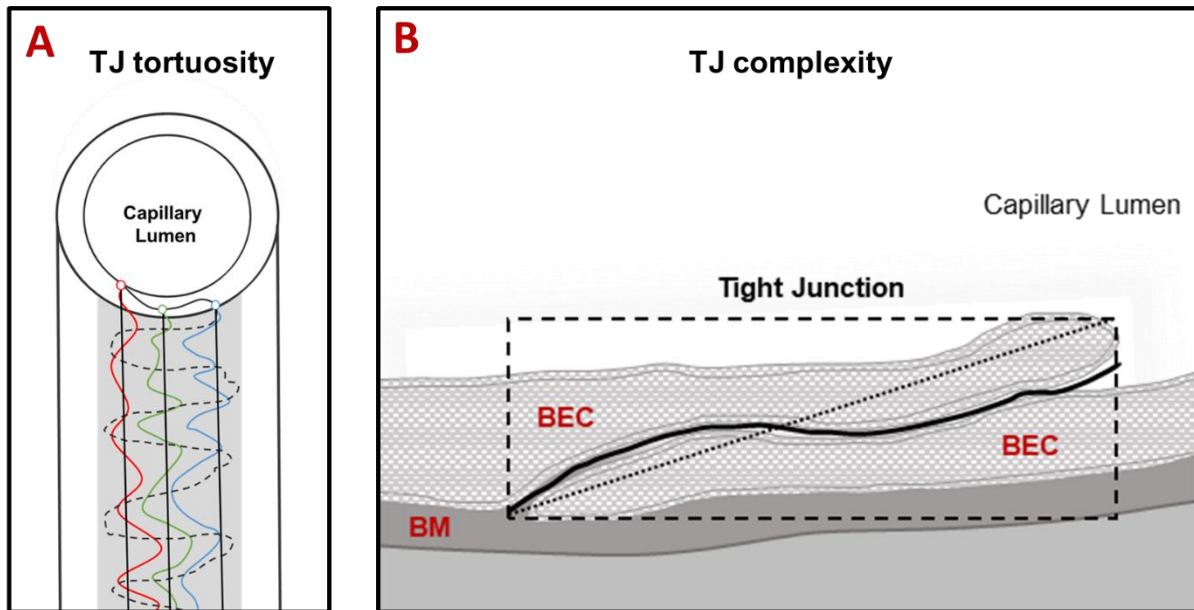


Figure 7. TJ measurements in 3D modelled cortical and hippocampal capillaries.

A) TJ tortuosity was calculated alongside the capillary as the average of three reference points in the actual TJ: abluminal (red), middle part of the TJ (green) and basolateral (blue). Each one of them was referred to a straight line joining directly the first and last sections of the whole vessel. **B)** TJ complexity was calculated by dividing the total length of the TJ (black solid line) by the diagonal (dotted line) of the rectangle (dashed line) containing the height and the length of the TJ. The final value was obtained as average of the TJ complexity every 5 sections of the 3D capillary.

2.2.4 Statistical analysis

Every statistical data analysis was performed using GraphPad Software (Prism 8.2.0, La Jolla, USA). Shapiro-Wilk normality test and Q-Q plots were used to assess distribution of the data. Data from 2D analysis was analysed using an unpaired two-tailed Student's *t* test, whereas data from 3D analysis data was analysed using two-way ANOVA and Sidak's correction test. In all cases, significance is set at $p < 0.05$ and data are displayed as mean \pm SEM. The selection of appropriate statistical tests is indicated in each figure legend.

2.3 Results

2.3.1 Aged female mice show persistent vaginal cornification

Assessment of oestrous cycle via vaginal lavage indicated that the young female mice were cycling normally through the 4 phases (**Figure 8A – D**). Aged mice showed persistent vaginal cornification, which is observed as a lack of epithelial cells and is consistent with reproductive senescence (**Figure 8E**). Vaginal smears were similar between young female mice in dioestrous and aged female mice. Previous studies have shown that plasma oestrogen and progesterone concentrations in aged females are most closely matched in the dioestrous phase of the cycle (Felicio, Nelson, and Finch 1984; Nelson et al. 1982). Therefore, to avoid the effect of hormone-induced fluctuations on BBB gene expression leading to the interpretation of the data between young and aged mice, all young females were confirmed to be in dioestrous on the day of tissue collection.

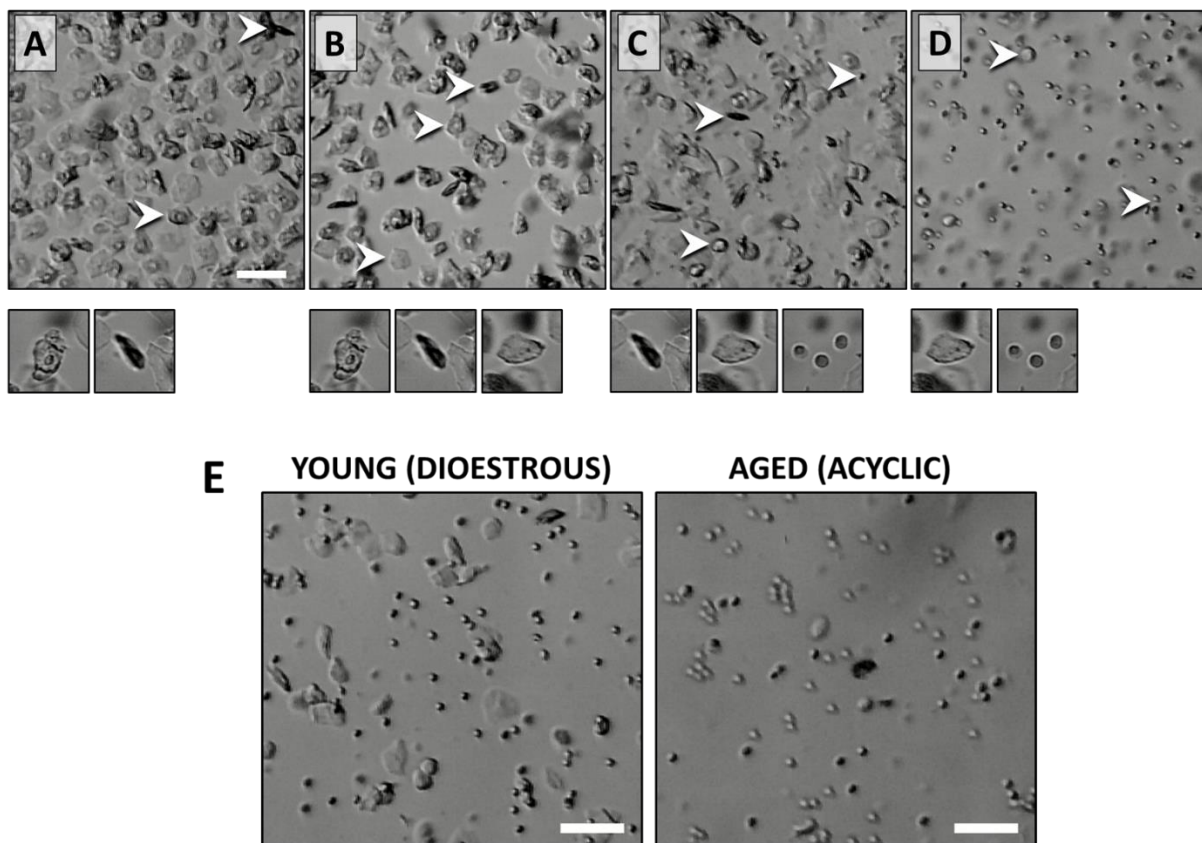


Figure 8. Classification of oestrous cycle stages in young and comparison with aged C57BL/6J female mice.

This classification is based on the amount and types of cells present in the vaginal smear. **A)** Pro-oestrous, presence of numerous nucleated epithelial cells (rounded and nucleated) and some cornified cells (needle-shaped, dark). **B)** Oestrous, presence of cornified cells with some nucleated and non-nucleated epithelial cells (similar shape to nucleated epithelial cells but without nucleus). **C)** Metoestrous, numerous cells including cornified and non-nucleated epithelial cells, leukocytes also start appearing. **D)** Dioestrous, mainly leukocytes with occasionally some non-nucleated cells. **E)** Young female mice in dioestrous showed a similar smear to aged acyclic female mice. Scale bars, 50 μm .

Progesterone and estradiol concentrations were measured using plasma samples from both young female mice in dioestrous and aged female mice. According to the results obtained by ELISA, no significant differences were observed in progesterone or estradiol levels between young and aged female mice (**Figure 9A**). Similarly, the sex steroid hormone ratio (Progesterone/Estradiol, P/E2) did not differ between young and female mice (**Figure 9B**).

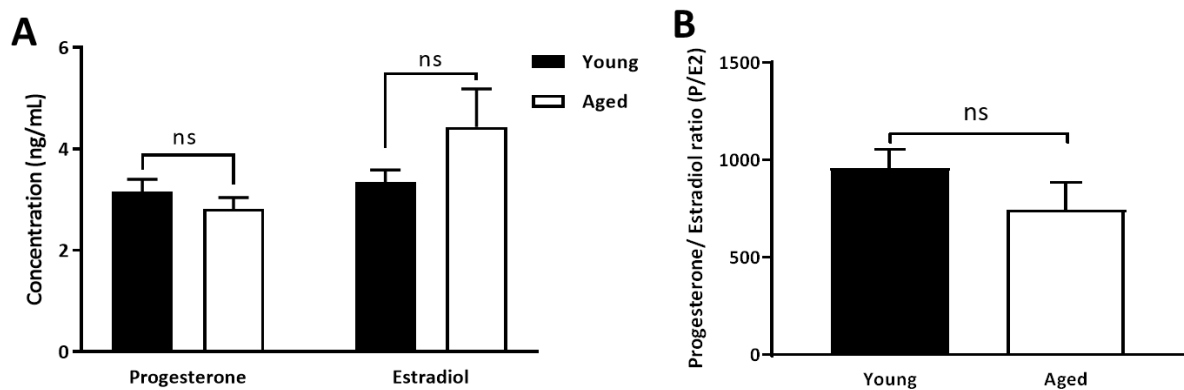


Figure 9. Progesterone and estradiol concentration and ratio (P/E2) in young and aged female C57BL/6J mice.

A) Progesterone and estradiol levels were measured in plasma samples by ELISA. No significant differences were observed between young and aged female mice for any of the hormones. **B)** P/E2 ratio was not significantly different between young and aged female mice. (n=6, Student's t test; ns, not significant).

2.3.2 Ultrastructural changes of female brain capillaries: preliminary 2D quantitative analysis

Analysis of 2D images was performed first, in order to measure age-induced changes at the female BBB, previously described in the ageing male mouse cortex.

2.3.2.1 Basement membrane (BM) thickness is increased in cortical aged capillaries

The BM was identified as a slightly electrodense layer located laterally to the endothelial cells (**Figure 10A**). A significant increase in the thickness of the BM was observed in aged capillaries compared to young capillaries (**Figure 10B**). The BM in aged capillaries was almost twice as thick as the BM of young capillaries.

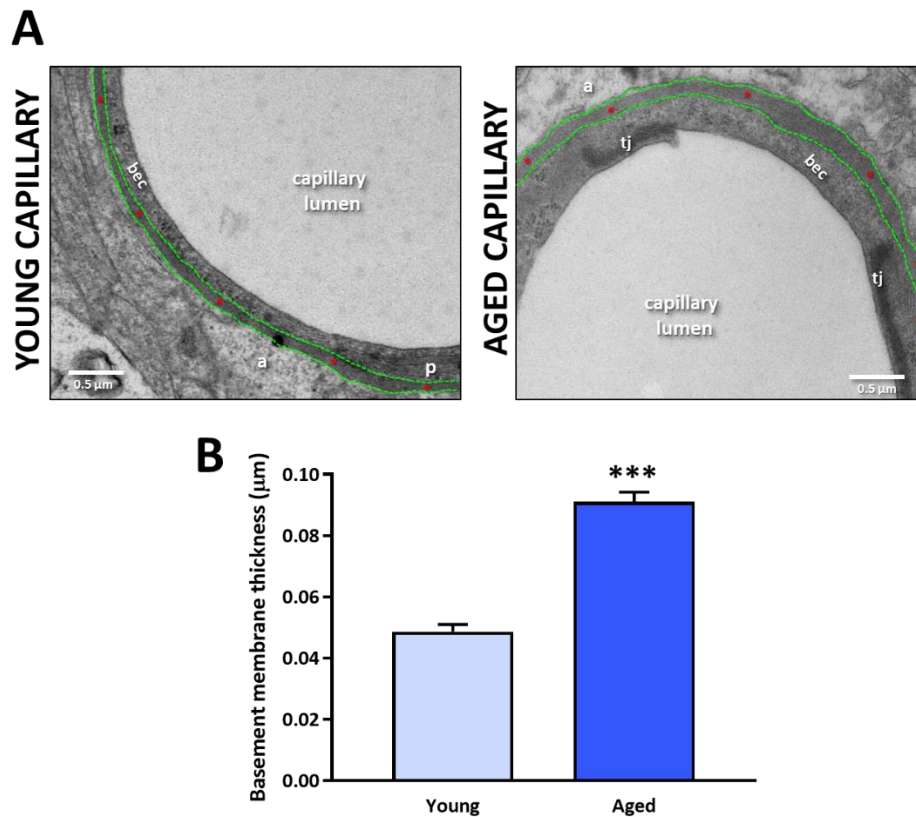


Figure 10. BM thickness in cortical capillaries of young and aged female C57BL/6J mice.

A) BM (green dotted line) was identified on TEM images of young and aged capillaries (Red stars (*): basement membrane; a: astrocyte; bec: brain endothelial cell; p: pericyte; tj: tight junction). **B)** Quantification of BM thickness showed that BM of aged capillaries was almost twice as thick as the BM of young mice. (n=3/age group, Student's t test; ***, $p < 0.001$).

2.3.2.2 Mitochondria of aged BECs in cortex are smaller and less numerous

Mitochondria were observed in TEM images as electrodense inclusions of diverse size within the cytoplasm (**Figure 11A**). In this analysis, BEC mitochondria area and number were measured with respect to total area of the cell. Both the number of mitochondria (**Figure 11B**)

and mitochondrial area (**Figure 11C**) were significantly decreased in aged BECs compared to young animals.

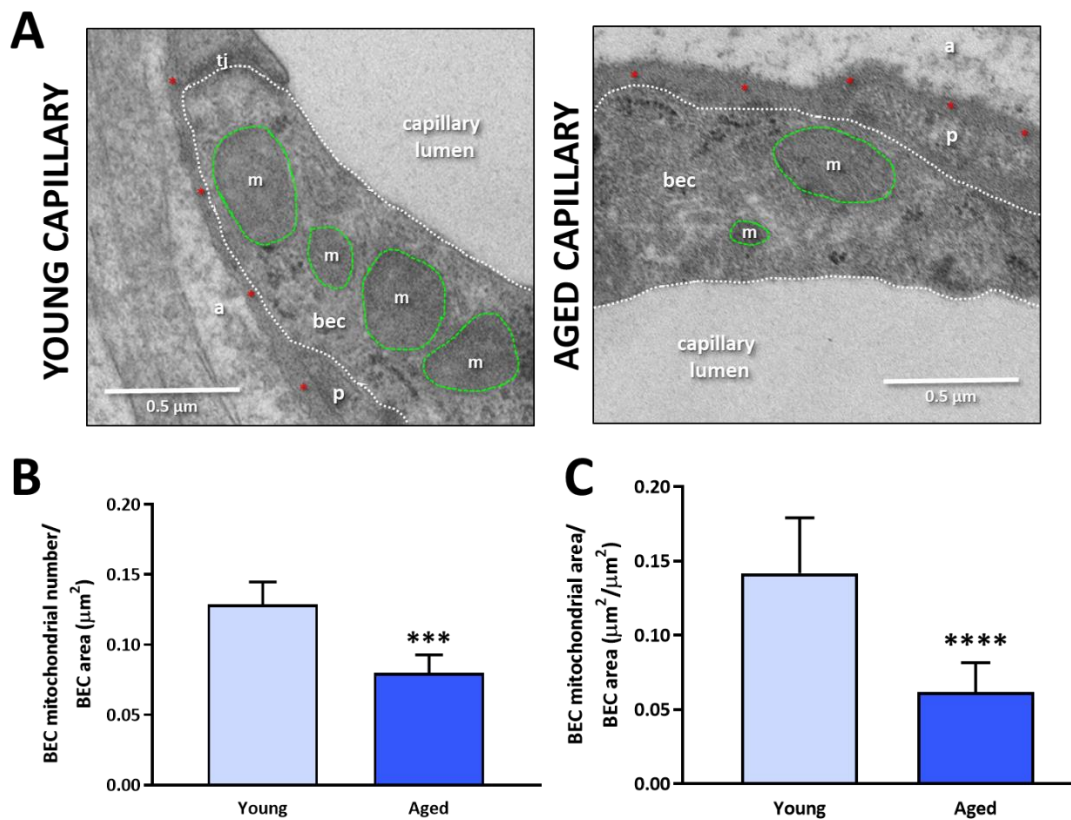


Figure 11. BEC mitochondria number and area in cortical capillaries of young and aged female C57BL/6J mice.

A) Mitochondria (green dotted line) were identified on TEM images of young and aged BECs (Red stars (*): basement membrane; white dotted line: brain endothelial cell area; a: astrocyte; bec: brain endothelial cell; m: mitochondria; p: pericyte; tj: tight junction). **B)** Number of mitochondria was significantly decreased in aged BECs. **C)** Mitochondrial area was significantly reduced in aged BECs. (n=3/age group, Student's t test; ***, $p < 0.001$; ****, $p < 0.0001$).

2.3.2.3 Mitochondria of aged pericytes are larger and more abundant

Pericyte mitochondria showed the same visual characteristics as described for BEC mitochondria (**Figure 12A**). However, in contrast to mitochondrial changes in BECs, the number of mitochondria in pericytes of aged capillaries was significantly higher than in pericytes at capillaries of young mice (**Figure 12B**). Mitochondrial area was also significantly greater in aged pericytes compared to those in young capillaries (**Figure 12C**).

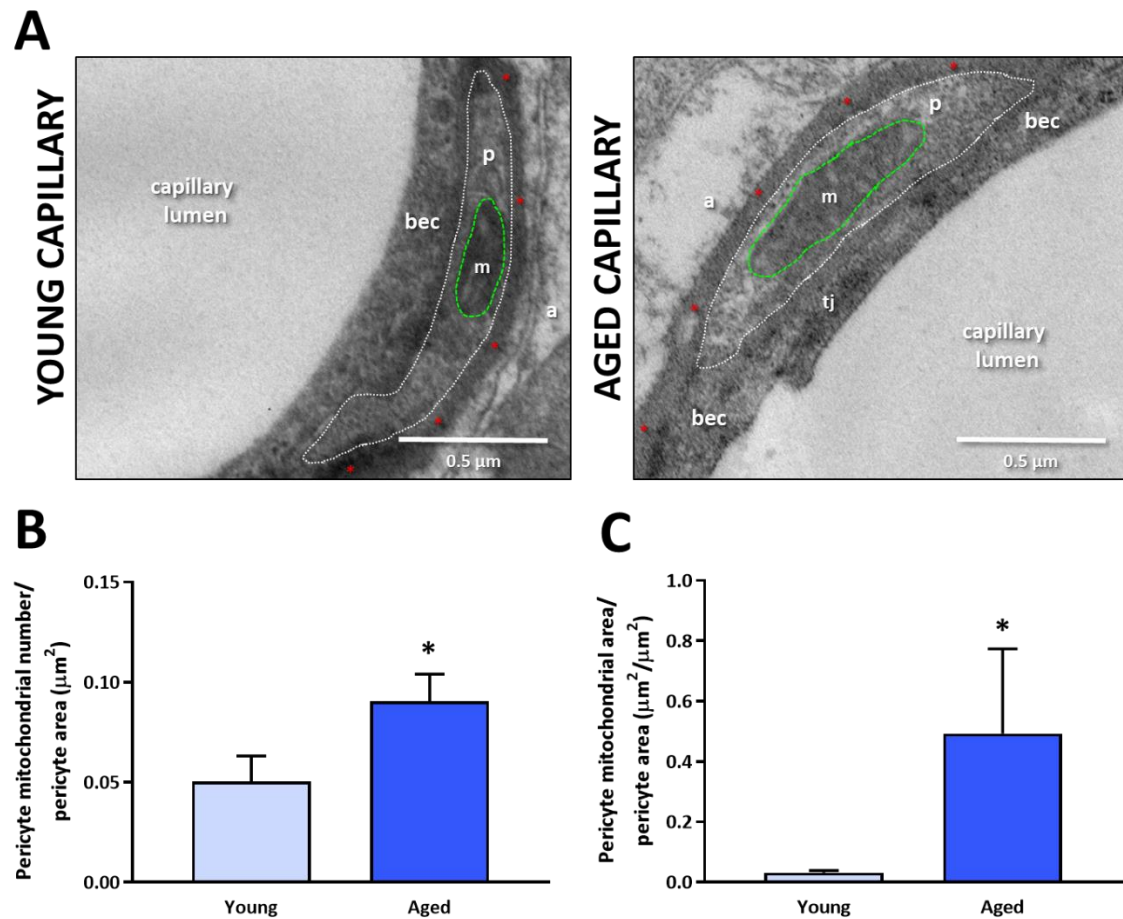


Figure 12. Pericyte mitochondria number and area in cortical capillaries of young and aged female C57BL/6J.

A) Mitochondria (green dotted line) were identified on TEM images of young and aged pericytes (Red stars (*): basement membrane; white dotted line: pericyte cell area; a: astrocyte; bec: brain endothelial cell; m: mitochondria; p: pericyte; tj: tight junction). **B)** Number of mitochondria was significantly increased in aged pericytes. **C)** Mitochondrial area was significantly greater in aged pericytes. (n=3/age group, Student's t test; *, $p < 0.05$).

2.3.2.4 Contact between BECs and pericytes is not changed in ageing

To determine whether the degree of contact between pericytes and BECs was altered by ageing, the percentage of pericytic coverage over BEC diameter was calculated (**Figure 13A**). No significant differences in the degree of pericyte – BEC contact between young and aged mice were found (**Figure 13B**).

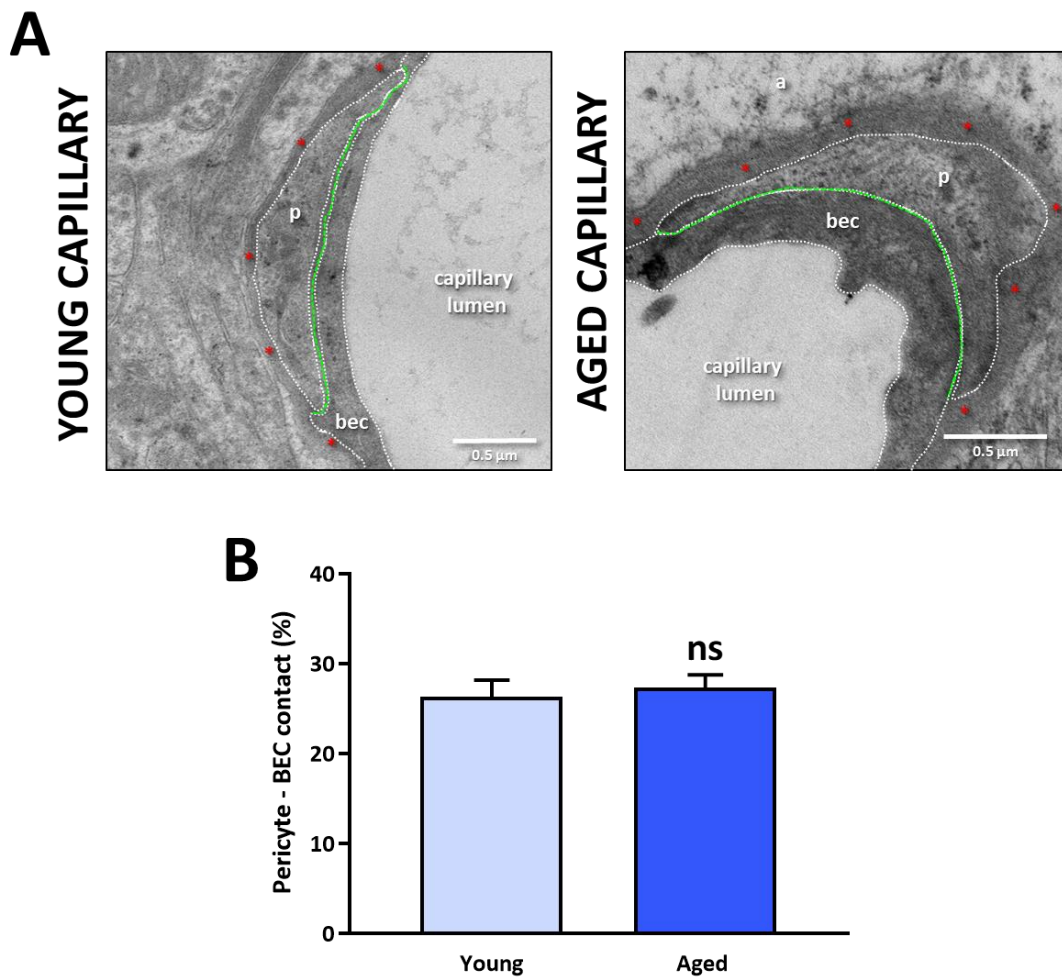


Figure 13. Pericyte – BEC contact in cortical capillaries of young and aged female C57BL/6J mice.

A) Pericyte – BEC contact (green dotted line) was identified on TEM images of young and aged capillaries. (Red stars (*): basement membrane; white dotted lines: pericyte and brain endothelial cell area; a: astrocyte; bec: brain endothelial cell; p: pericyte). **B)** Quantification of pericyte – BEC contact showed that this contact was not changed in aged capillaries. (n=3/age group, Student’s T test; ns, not significant).

2.3.2.5 Contact between BM and astrocytes is increased in ageing

Astrocytic end-feet appear like a large light structure that surrounds BECs, pericytes and BM. They are variable in size and can be traced across the brain parenchyma. In this analysis, only the astrocytic structures included in an area of 1 μm away of the vessel were traced. Image J was used to trace the area of both astrocytes and BM and the contact between both components was calculated as the percentage of area per area (**Figure 14**). According to

these results, contact of BM and astrocytes is slightly but significantly increased in aged capillaries over young ones.

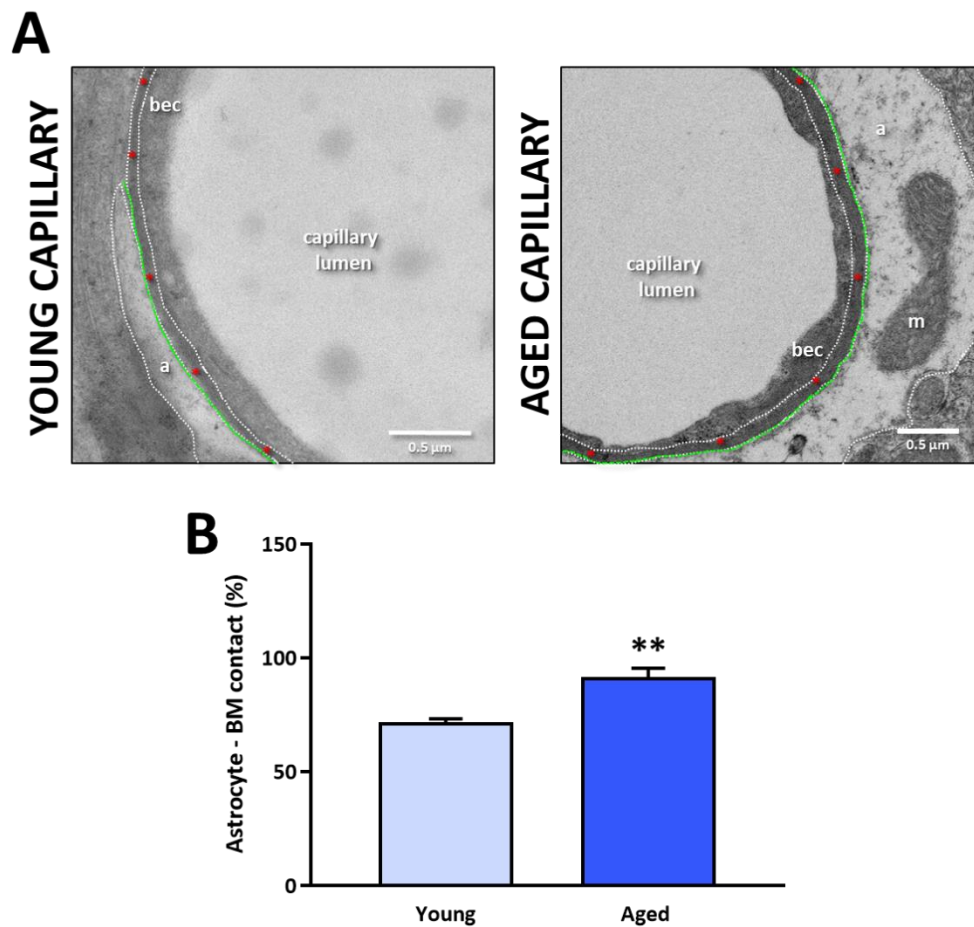


Figure 14. BM – astrocyte contact in cortical capillaries of young and aged female C57BL/6J mice.

A) Astrocyte – BM contact was identified on TEM images of young and aged capillaries. (Red stars (*): basement membrane; white line: astrocyte and BM area; a: astrocyte; bec: brain endothelial cell; m: mitochondria; p: pericyte). **B)** Astrocyte – BM contact was shown to be significantly increased in aged capillaries. (n=3/age group, Student's t test; **, $p < 0.01$).

2.3.3 Ultrastructure of cortical and hippocampal brain capillaries: A 3D quantitative analysis

2.3.3.1 BM is thicker in aged capillaries of cortex and hippocampus

After 3D reconstruction, the BM was visible as a continuous layer covering the whole microvessel (**Figure 15A and B**). Similar to the findings from the 2D images, a significant increase in BM thickness was observed in aged capillaries compared to young capillaries, in both the cortex and hippocampus (**Figure 15C**). In both cases, BM was almost twice as thick in aged capillaries than in young capillaries. Regional comparisons of BM thickness between the cortex and hippocampus did not show any significant differences in either age group.

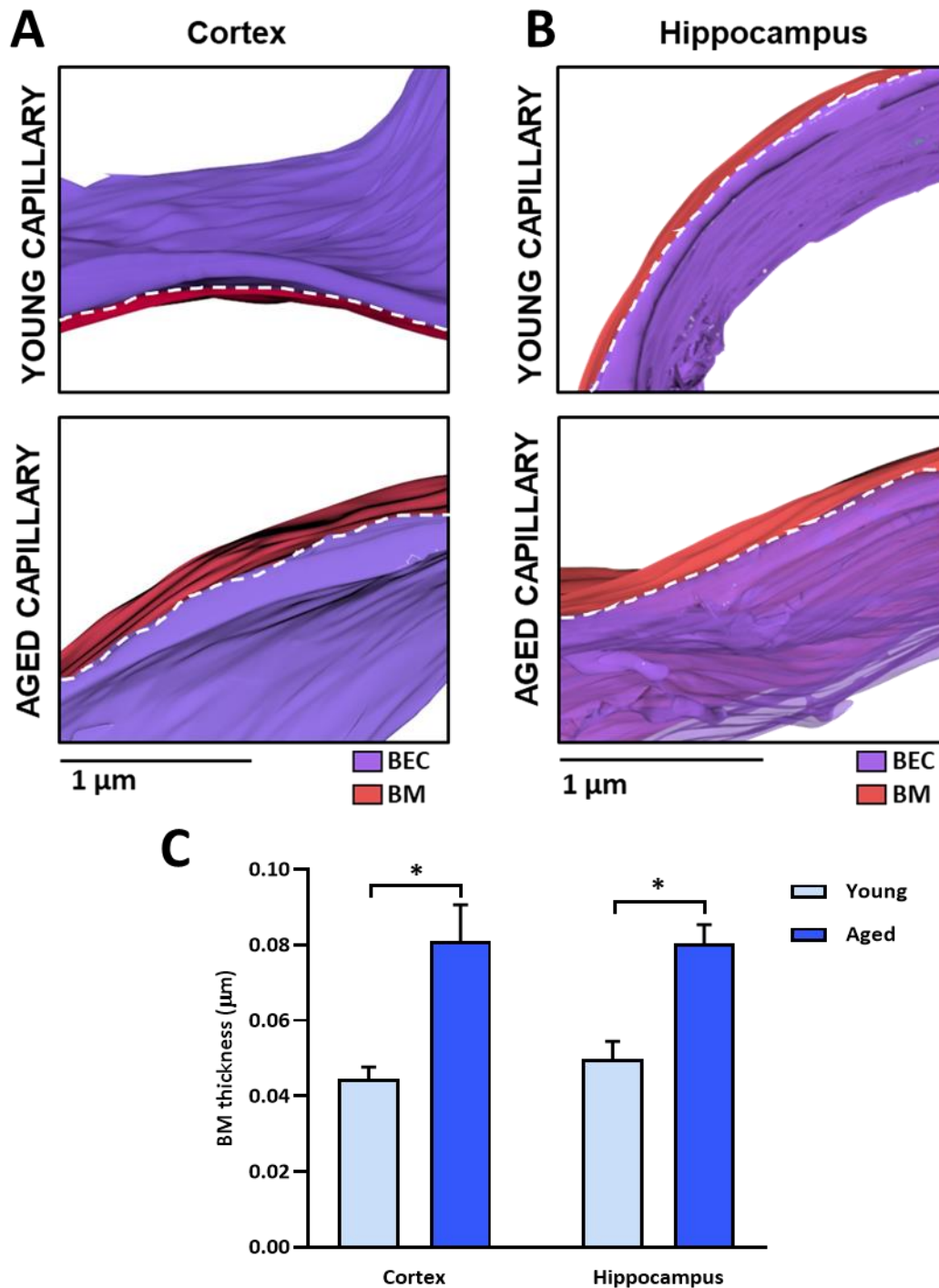


Figure 15. BM thickness in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

BM was identified in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Cortical aged capillaries showed a significant increase in BM thickness when compared to young capillaries from the same region. Similarly, BM thickness was significantly higher in aged capillaries compared to young capillaries in the hippocampus, although the fold-change is smaller. In contrast, no regional differences were observed in BM thickness. (n=3/age group, Two-way ANOVA and Sidak's correction test; *, $p < 0.05$).

2.3.3.2 Mitochondrial number and volume are not changed in cortical or hippocampal BECs

The 3D reconstruction showed mitochondria as rounded inclusion bodies within the cytoplasm of the endothelial cell (**Figure 16A and B**). Interestingly, the mitochondria grouped together alongside the length of the vessel. In contrast to the findings from the 2D analysis, mitochondrial number did not show significant differences between aged and young BECs in cortex or hippocampus (**Figure 16B**). Similarly, BEC mitochondrial volume was not significantly changed in aged capillaries compared to young capillaries in cortex and hippocampus (**Figure 16C**). No differences were observed either when comparing both regions in age-matched individuals.

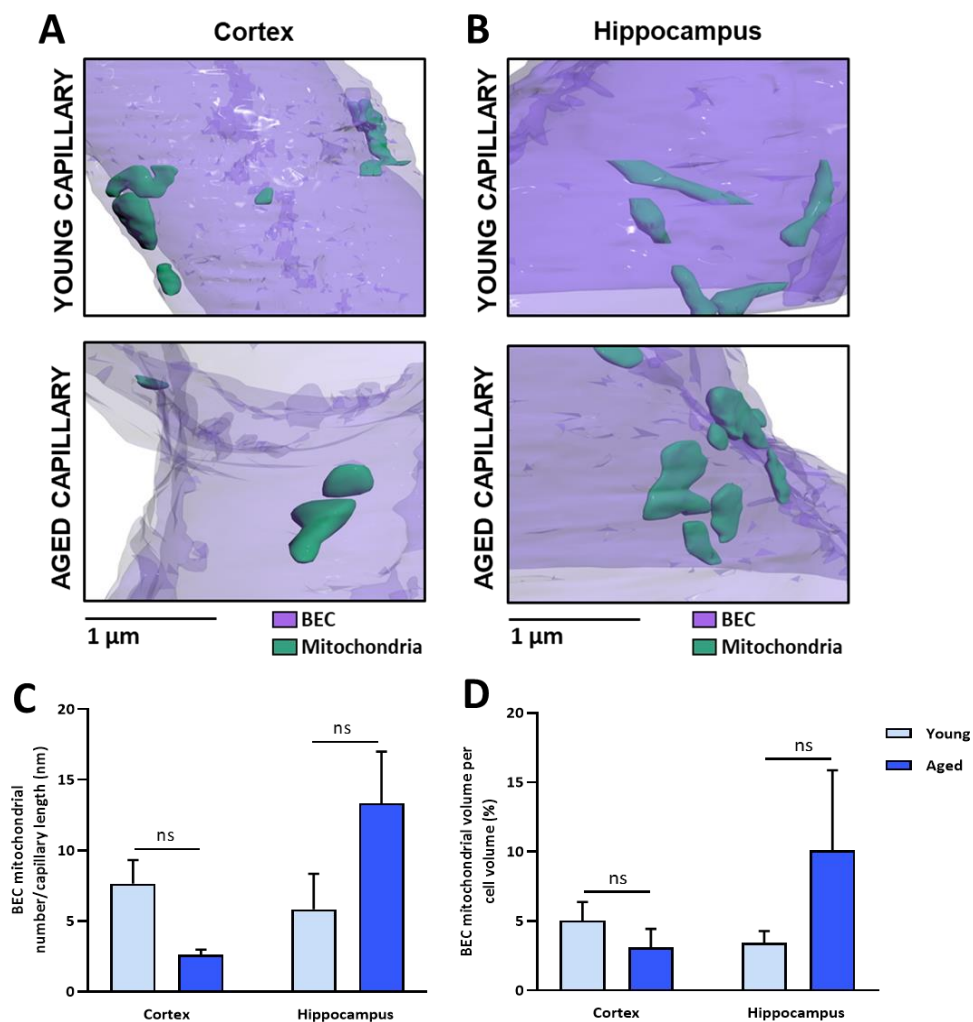


Figure 16. BEC mitochondria structural analysis in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

Mitochondria were identified as rounded inclusions within the BEC cytoplasm in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Mitochondrial number was not significantly changed in aged capillaries from cortex or hippocampus when compared to young capillaries from the same region. **D**) Mitochondrial volume did not show significant changes in aged capillaries when compared to young capillaries in cortex or hippocampus. In addition, no regional differences were observed in mitochondrial number or volume when comparing age-matched individuals. (n=3/age group, Two-way ANOVA and Sidak's correction test; ns, not significant).

2.3.3.3 BEC pseudopod number and volume are increased in aged cortical capillaries

When comparing young and aged capillaries, filamentous pseudopod-like structures were observed to protrude from the BECs into the lumen of the vessels. In 3D reconstructions, pseudopods were observed on the luminal surface of the vessels and show different sizes (**Figure 17A and B**).

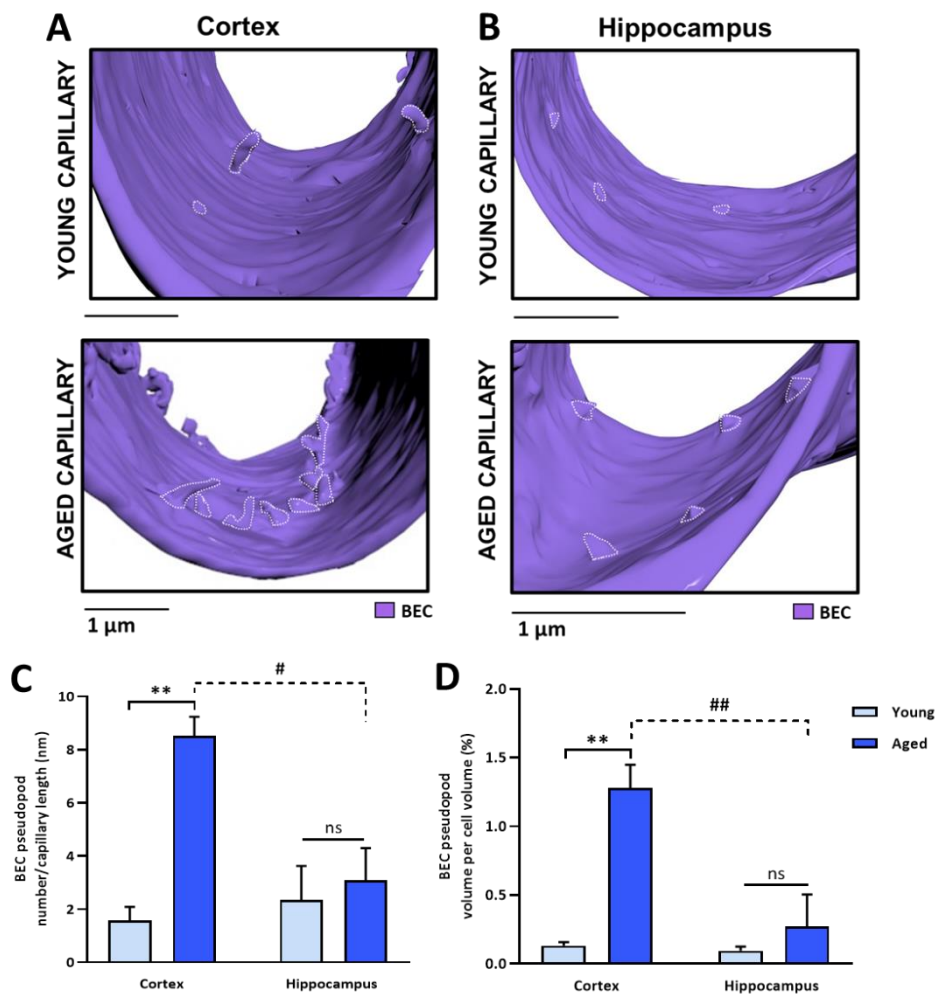


Figure 17. BEC pseudopod structural analysis in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

Pseudopods were identified as protrusions from the endothelial cytoplasm into the vessel lumen in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Pseudopod number was significantly increased in aged cortical capillaries compared to young cortical capillaries, whereas no changes were observed between aged and young mice in hippocampus. In addition, Pseudopod number was significantly greater in aged capillaries from cortex than in aged capillaries from hippocampus. **D**) Volume of pseudopods was significantly higher in cortical capillaries from aged animals compared to young cortical capillaries. In contrast, no changes in pseudopod volume were observed between aged and young mice in hippocampus. Additionally, pseudopod volume was significantly greater in aged capillaries from cortex than in aged capillaries from hippocampus. (n=3/age group, Two-way ANOVA and Sidak's correction test; **, ##, p<0.01; #, p<0.05; ns, not significant).

Both pseudopod volume and number were significantly increased in aged cortical BECs when compared to endothelial cells from young cortical capillaries (**Figure 17C and D**). Pseudopod number was 4-fold higher in aged BECs than in young BECs, whereas pseudopod volume was 10-fold higher than the volume in young BECs. In the hippocampus, no significant differences were observed in pseudopod volume or number between young and aged vessels. Regional comparisons showed that both pseudopod number and volume were significantly higher in aged cortical capillaries than in aged hippocampal capillaries.

2.3.3.4 Mitochondrial volume is increased in aged pericytes of cortex and hippocampus

As mentioned above, the 3D reconstruction showed mitochondria as rounded inclusions within the cytoplasm of pericytes (**Figure 18A and B**). Assessment of the number of mitochondria in pericytes found that it was not changed in aged pericytes of cortical or hippocampal capillaries (**Figure 18C**). In contrast, mitochondrial volume was significantly increased in aged pericytes of cortex and hippocampus in comparison to young pericytes of the respective regions (**Figure 18D**). Volume of pericytic mitochondria was almost 5-fold higher in aged cortical capillaries when compared to young cortical capillaries, whereas almost twice as many mitochondria were observed in aged hippocampal capillaries compared to young hippocampal capillaries. It was also noted that, in some cases, pericyte mitochondria formed tubular networks in aged pericytes of the cortex. Regional comparisons did not show significant differences in number or volume of pericytic mitochondria between cortical and hippocampal capillaries at any age.

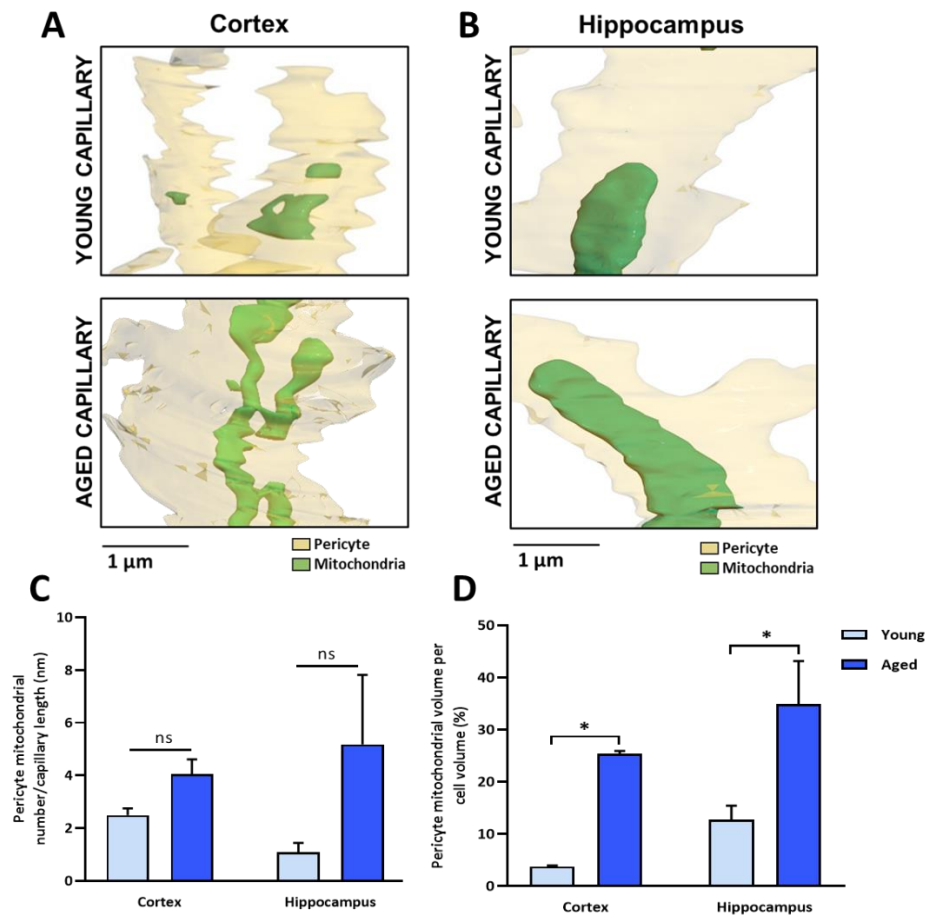


Figure 18. Pericyte mitochondria structural analysis in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

Mitochondria were identified as rounded inclusions within the pericyte cytoplasm in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Number of mitochondria did not show significant differences between aged and young capillaries in cortex or hippocampus. **D**) Volume of mitochondria was significantly increased in aged capillaries of cortex and hippocampus when compared to their young counterparts. In some cases, mitochondria were observed to form tubular networks in aged pericytes. In contrast, regional comparisons did not show significant changes in pericytic mitochondrial volume or number between cortical and hippocampal capillaries at any age. (n=3/age group, Two-way ANOVA and Sidak's correction test; *, p<0.05; ns, not significant).

2.3.3.5 Contact between pericytes and BECs is increased in cortical aged capillaries

In the 3D reconstructed capillaries, pericytes appeared as projections that intermittently spread over the vessel wall (**Figure 19A and B**).

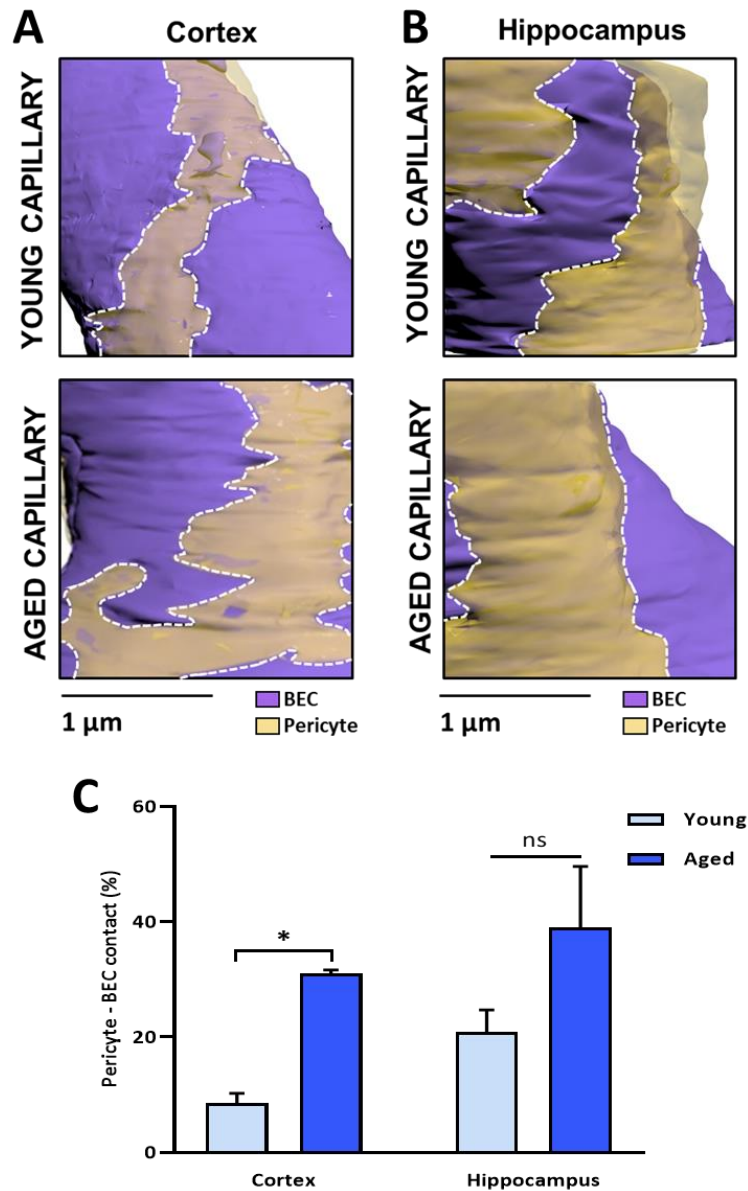


Figure 19. Structural analysis of the contact between pericytes and BECs in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

Pericyte projections were identified intermittently spread over the vessel wall in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Percentage of pericyte – BEC contact was significantly increased in cortical aged capillaries compared to cortical young capillaries. In contrast, pericyte – BEC contact did not change in hippocampal capillaries with ageing. Regional comparisons did not show significant changes in pericyte – BEC between cortical and hippocampal capillaries at any age. (n=3/age group, Two-way ANOVA and Sidak’s correction test; *, $p < 0.05$; ns, not significant).

Analysis of the degree of contact between BEC and pericytes showed a significant increase in the percentage of pericyte – BEC contact in aged cortical capillaries (**Figure 19C**). However, no significant differences were observed in pericyte – BEC contact in hippocampal

capillaries of young and aged female mice. Similarly, regional comparison showed no significant differences in pericyte – BEC contact in cortical versus hippocampal capillaries in either young or aged mice.

2.3.3.6 Contact between astrocytes and capillary wall is not changed in age

When capillaries were reconstructed, astrocytic end-feet were observed as large projections that ensheathed the capillary and established contact with the BM (**Figure 20A and B**). Quantification of contact between astrocytes and BM showed no significant differences between aged and young capillaries in cortex or hippocampus (**Figure 20C**). Regional comparisons did not show significant differences in astrocyte end-feet coverage between cortical and hippocampal capillaries in young or aged mice.

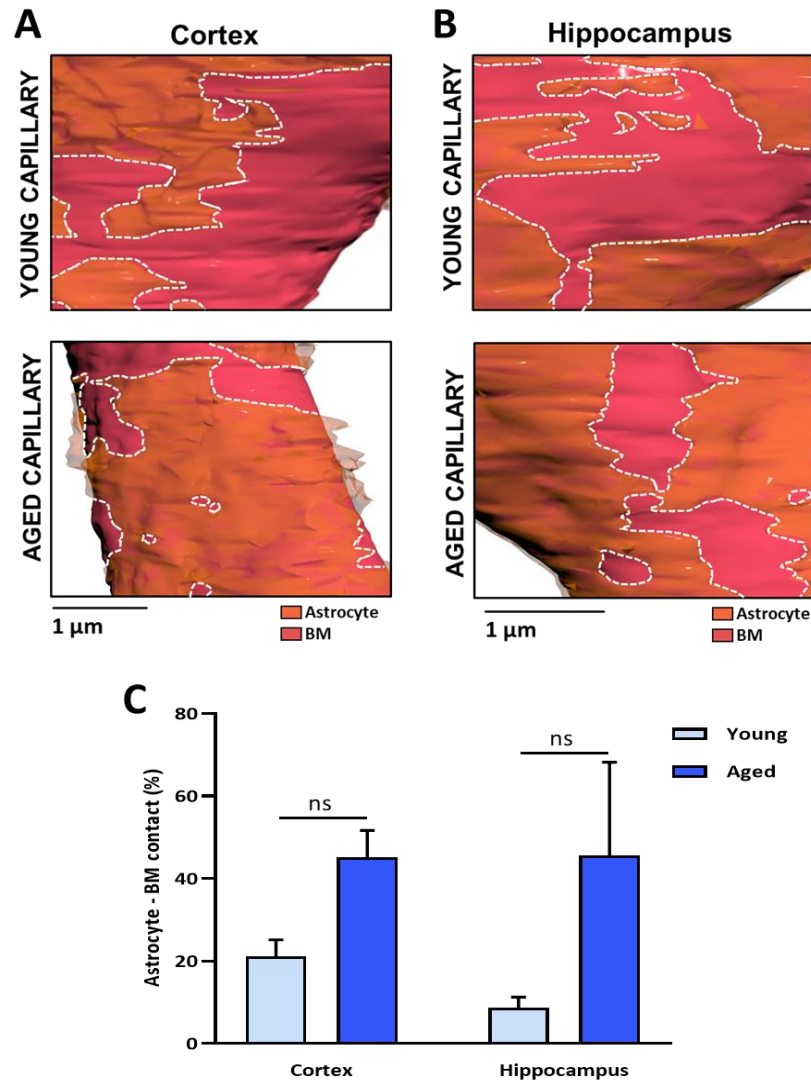


Figure 20. Analysis of the contact between astrocytes and BM in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

Astrocytic end-feet were identified as large projections ensheathing the vessel and establishing contact with the BM in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) Percentage of astrocyte – BM contact was not significantly changed in aged capillaries compared to young capillaries in cortex or hippocampus. Similarly, astrocyte – BM contact did not show regional differences in any age when comparing cortical and hippocampal capillaries. (n=3/age group, Two-way ANOVA and Sidak's correction test; ns, not significant).

2.3.3.7 Tight junction tortuosity is increased in aged cortical capillaries

TJ are visible alongside the capillary in the 3D model where two BECs overlap (**Figure 21A and B**). Analysis of TJs showed a significant increase in tortuosity in aged cortical capillaries compared to cortical young capillaries (**Figure 21C**). No differences were observed

between young and aged capillaries in the hippocampus. In addition, TJ tortuosity was significantly higher in aged capillaries of the cortex compared to aged capillaries of the hippocampus.

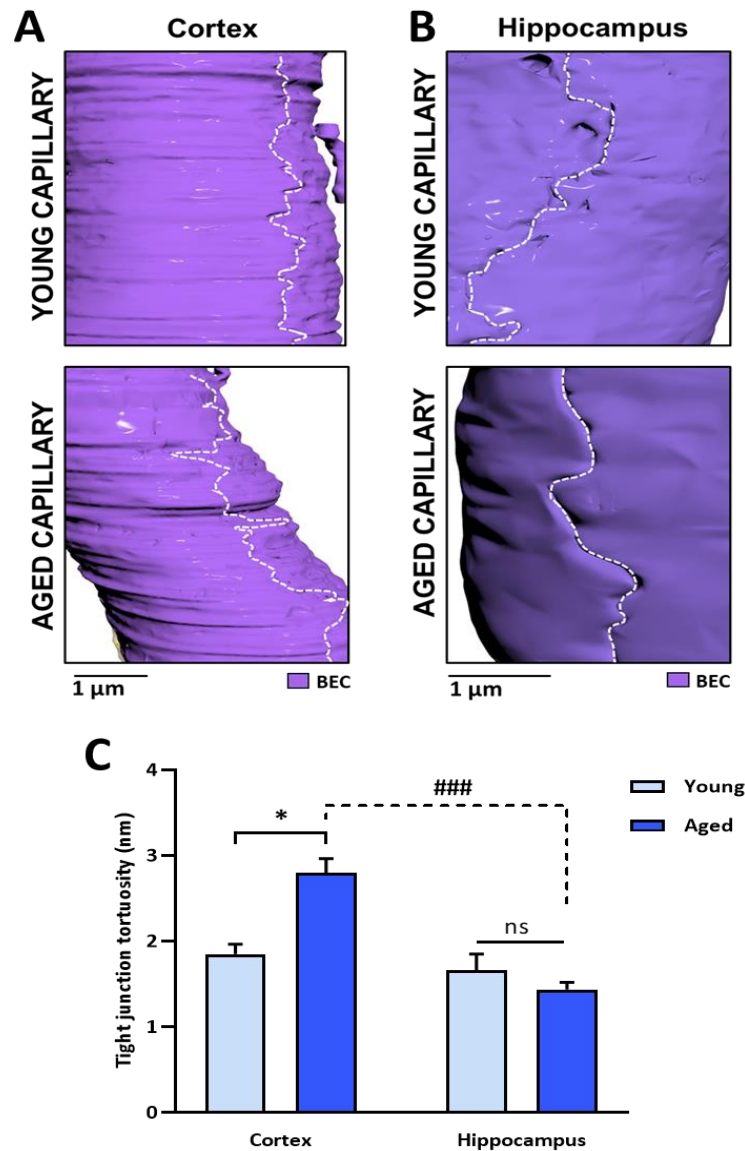


Figure 21. TJ tortuosity analysis in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

TJs were identified alongside the vessel where two BECs overlap in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) TJ tortuosity was significantly higher in aged cortical capillaries compared to young cortical capillaries, whereas no changes were observed in hippocampal capillaries. Regional comparisons showed a significantly higher TJ tortuosity in aged cortical capillaries in comparison to aged hippocampal capillaries. (n=3/age group, Two-way ANOVA and Sidak's correction test; ###, $p < 0.001$; *, $p < 0.05$; ns, not significant).

Regarding TJ complexity, understood as length, it can be observed on the edges of the reconstructed vessel or in each TEM section (transversal view) (**Figure 22A and B**). Our results showed no significant differences between young and aged capillaries in either cortex or hippocampus (**Figure 22C**). However, when comparing both regions, a small but significant increase in TJ complexity was observed in aged hippocampal capillaries versus aged cortical capillaries.

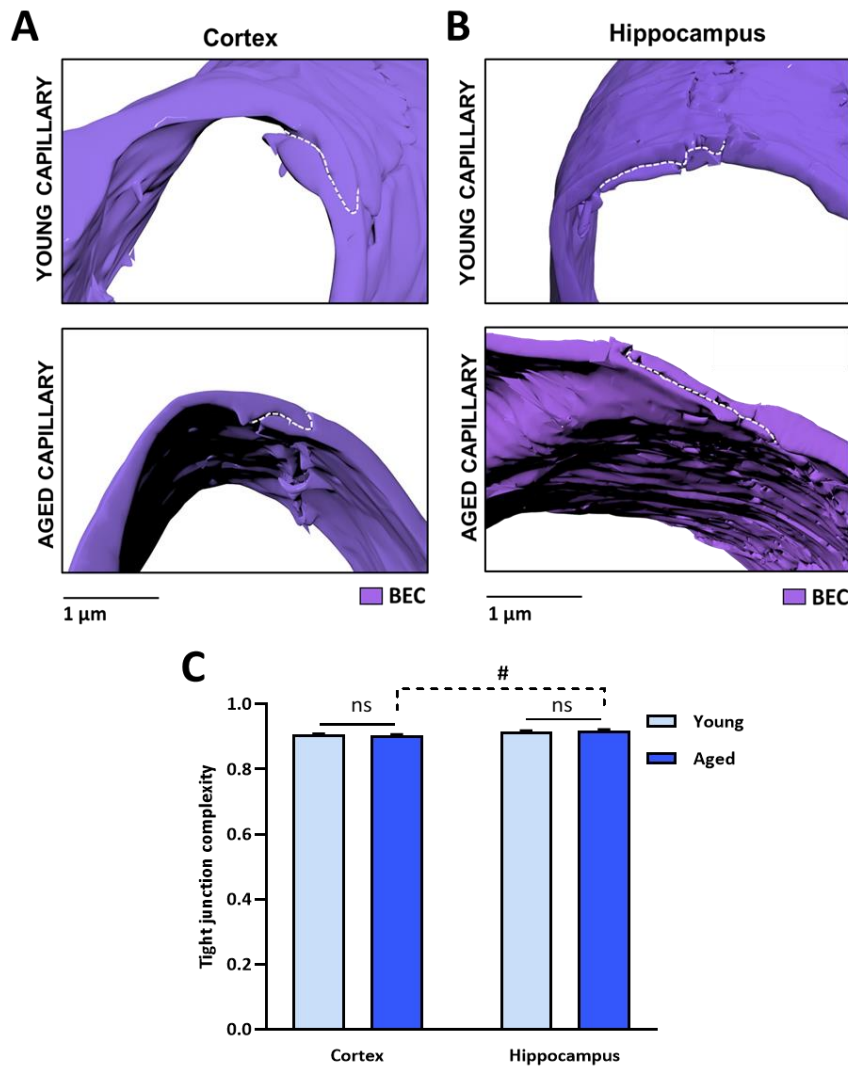


Figure 22. TJ complexity measured in 3D reconstructed cortical and hippocampal capillaries of young and aged female C57BL/6J mice.

To analyse complexity, TJs were identified at the edges of the vessel (transversal section) in 3D reconstructed capillaries of young and aged mice, in both **A**) cortex and **B**) hippocampus. **C**) TJ complexity was not significantly changed by age in cortical or hippocampal capillaries. Regional comparisons showed a small but significant increase in TJ complexity in aged hippocampal capillaries versus aged cortical capillaries. (n=3/age group, Two-way ANOVA and Sidak's correction test; #, $p < 0.05$; ns, not significant).

2.4 Discussion

In the present study, structural features were analysed in cerebral capillaries from young and aged C57BL/6J female mice, in order to assess age-related changes in the female BBB ultrastructure. Sexual steroid hormone levels were measured by ELISA, and P/E2 was not significantly different between young and aged female mice. Preliminary structural analysis was performed in 2D TEM images of cortical capillaries only, whereas a further 3D reconstruction analysis was performed in both cortical and hippocampal capillaries. In general, 2D and 3D analyses appear to have some differences although several age-related changes were observed in cortical capillaries. In addition, the region comparison results showed a higher impact of ageing on cortical capillaries compared to hippocampal capillaries. A summary of the results can be found in **Table 2**.

Table 2. Summary of ultrastructural changes in the ageing female BBB assessed by TEM.

	Young vs Aged Female Mice			Cortex vs Hippocampus
	Cortex		Hippocampus	
<i>Structural Features</i>	<i>Preliminary 2D Analysis</i>	<i>3D Reconstruction Analysis</i>	<i>3D Reconstruction Analysis</i>	<i>3D Reconstruction Analysis</i>
BM thickness	Higher in aged capillaries	Higher in aged capillaries	Higher in aged capillaries	No difference
BEC mitochondria (Number and area/volume)	Lower in aged capillaries	No difference	No difference	No difference
BEC Pseudopods (Number and area/volume)	N/A	Higher number and volume in aged capillaries	No difference	Higher number and volume in aged cortical capillaries
Pericyte mitochondria (Number and area/volume)	Higher in aged capillaries	Higher volume in aged capillaries No difference in number	Higher volume in aged capillaries No difference in number	No difference

Pericyte coverage over BECs	No difference	Higher in aged capillaries	No difference	No difference
Astrocyte coverage over BM	High in aged capillaries	No difference	No difference	No difference
Tight junction tortuosity	N/A	Higher in aged capillaries	No difference	Higher in aged cortical capillaries
Tight junction complexity	N/A	No difference	No difference	Lower in aged cortical capillaries

The process of ageing in females is undoubtedly related to reproductive senescence and changes in sexual hormone levels, in humans, rodents and non-primate human (Maffucci and Gore 2006; Mobbs, Gee, and Finch 1984). However, the role of certain sex steroid hormones, such as oestrogen, progesterone or testosterone in BBB modulation with ageing is a matter of controversy (Lutescu et al. 2007; Sohrabji 2005; Viña et al. 2013). In the current study, reproductive senescence was confirmed by determination of acyclicity, using the protocol from Byers and colleagues (Byers et al. 2012). To determine progesterone and oestrogen (estradiol) levels, plasma samples from young and aged female mice in dioestrous were assessed by ELISA. Ratio between progesterone and estradiol (P/E2) did not change between young and aged female mice. However, these results are not consistent with previous studies that showed young P/E2 ratios to be closer to 50/1 – 300/1 and also described an age-related increase in the ratio itself (Nelson et al. 1981). These striking differences may be due to the use of immunoassay-based techniques as ELISA. Indeed, even though ELISA assays have been suitable for hormone level measurements in plasma (Elder et al. 1987; Relave et al. 2007), several studies have reported their questionable specificity, especially at lower concentrations (Haisenleder et al. 2011; Huhtaniemi et al. 2012). Ideally, gas chromatography-tandem mass spectrometry techniques would be a fitting option to measure hormone levels in plasma, as they have been reported to show high specificity even for low postmenopausal hormone concentrations (Lee et al. 2006; Nilsson et al. 2015), however, they required higher specimen volume and cost.

Structural 2D analysis was performed only in cortical capillaries as a preliminary study in order to confirm previously described age-induced alterations in the male BBB ultrastructure. Additionally, structural 3D analysis was performed in capillaries from cortex

and hippocampus, since both regions have been reported to be altered in brain ageing and age-related BBB dysfunction in both humans and rodents (Montagne et al. 2015; Pelegrí et al. 2007; Viggars et al. 2011). Interestingly, despite some features being similarly changed when assessed either by 2D or 3D technique (e.g. BM thickness or area/volume of pericytic mitochondria), some other structural characteristics did not correlate when investigated by the two approaches and appeared to highlight technical differences (e.g. BEC mitochondrial number and area/volume, pericyte – BEC coverage or astrocyte – BM coverage). The main differences between the two studies are the addition of cellular and subcellular interactions in space and the higher in-depth analysis achieved by the 3D reconstruction, which become useful when analysing complex and interrelated structures in brain microvessels (Mathiisen et al. 2010). The lack of depth in the 2D analysis might hinder the assessment of certain features as it happened in the current study with BEC pseudopods or TJ tortuosity and complexity. Similarly, the three-dimensional reconstruction could facilitate the identification of alterations that did not show changes in the 2D analysis, such as pericyte coverage or the observation of pericytic mitochondria occasionally forming tubular networks. However, the experimental number might also contribute to these differences. Although the 3D analysis shows a higher resolution, it is limited by the processing and its time-consuming protocol. Therefore, future analyses should attempt to increase the number of vessels reconstructed, in order to enhance the power of the experiment and determine whether the non-altered features observed are truly unchanged. Nonetheless, a combination of both, 2D and 3D studies, might be a much better strategy in those cases in which the experimental number is limiting the experimental design.

One of the most obvious alterations observed in the 2D analysis of cortical capillaries and the 3D analysis of both cortical and in hippocampal capillaries, was the increased BM thickness with ageing. In agreement with previous studies, BM thickness in aged microvessels in both cortex and hippocampus was double that of young animals (Ceafalan et al. 2019). The BM is an essential element of the BBB, enclosing pericytes and interacting via adhesion molecules with the astrocytic end-feet (Correale and Villa 2009). It has already been reported that cerebrovascular BM undergoes a series of molecular and morphological alterations during normal ageing and in neurodegenerative diseases in humans and rodents, with no particular changes due to sex (Alba et al. 2004; Farkas et al. 2000, 2006; A. W. J. Morris et al. 2014). Increased thickness and stiffness of the BM has been reported to happen in humans and rodents and is linked to changes in the proportion of extracellular matrix proteins, such as collagen IV and laminins (Candiello et al. 2010; Ceafalan et al. 2019; Uspenskaia et al. 2004). Apart from

the potential effect on blood flow, higher thickness and stiffness appear to be related to the accumulation of unwanted proteins in the brain, hindering clearance mechanisms in the mouse cerebral vasculature (Hawkes et al. 2011). Indeed, interaction of BM components with proteins such as amyloid- β ($A\beta$) have been reported to contribute to cerebrovascular pathology leading to AD in humans (Shimizu et al. 2009). Therefore, age-induced alterations in BM consequently lead and contribute to neurodegeneration, not only in AD but also in PD, where they are more enhanced than in normal ageing (Farkas et al. 2000).

Mitochondrial changes and impairment have been described in the aged BBB in several studies and different species (e.g. mouse, monkey, human), mainly associated with oxidative stress and pro-inflammatory processes in the cerebral cortex (Burns et al. 1979; Enciu et al. 2013; Sure et al. 2018). Indeed, ageing has been linked to endothelial cell dysfunction via production of ROS which also leads to a reduced mitochondrial number (Brandes et al. 2005). Mitochondrial morphology depends on the balance of fusion and fission mechanisms, which contribute to modulate respiratory function, mitochondrial DNA integrity or cell death (Aerts et al. 2008; Leadsham and Gourlay 2010). In addition, some studies have reported age-induced effects on fusion/fission dynamics, which might contribute to changes in morphology and accumulation of damage in the aged mitochondria (Jendrach et al. 2005; Scheckhuber et al. 2011). In the current study, mitochondrial morphology has been assessed in BECs and pericytes, and was confirmed to change in ageing, although with some controversy between the techniques used. Age-related loss of BEC mitochondria has been supported by several studies that described a decrease in mitochondrial number and volume in BECs of aged male mice, primates and ageing human endothelial cells *in vitro* (Burns et al. 1979; Hicks et al. 1983; Jendrach et al. 2005). These findings are in agreement with changes observed in cortical capillaries in our 2D analysis, but not with the results from 3D analysis in both cortex and hippocampus, which showed no significant differences in number or volume. By contrast, pericytic mitochondrial area and number were significantly increased in our 2D in aged cortical capillaries, although only volume, and not number, was significantly higher in both aged cortical and hippocampal capillaries in the 3D analysis. One possible explanation for differences in pericyte mitochondrial number could be the observation that in aged capillaries, mitochondria occasionally tend to form tubular networks, a feature that would interfere in the counting in 2D analyses. Nevertheless, these results are consistent with very early studies that reported a significant increase in pericytic mitochondrial volume during healthy ageing in male mice (Hicks et al. 1983; Mooradian 1988), and also with more recent studies that showed

swollen mitochondria in pericytes of Notch3 mutant transgenic mice in the context of cerebrovascular pathology (Gu et al. 2012). In addition, changes in mitochondrial shape have also been linked to mitophagy (mitochondrial autophagy) in ageing (Sun et al. 2015). In the context of mitophagy, mitochondria might undergo numerous fission events that lead to senescence and cell death (Scheckhuber et al. 2011) or, on the contrary, show reduced fission activity and display network-like morphology with the potential to maintain cell viability and postpone mitochondrial dysfunction and ageing (Gomes, Benedetto, and Scorrano 2011). Our results suggest that BEC and pericyte mitochondria are differently affected by senescence. Mitochondria are highly related to cell physiology, a statement that is even more important when considering the higher metabolic demand of the BBB (Haddad-Tóvolli et al. 2017). Modifications in mitochondrial morphology and function might highlight the higher sensitivity of the BBB to oxidant stressors, which together with decreased expression of antioxidant proteins and increased presence of ROS in ageing, promote cerebrovasculature damage (Grammas, Martinez, and Miller 2011). However, our observation of the formation of tubular networks of mitochondria in aged pericytes suggests that differential alterations in mitochondrial shape between BEC and pericytes mitochondria might be understood as pericytes being more resistant to age-induced damage than BECs in the female BBB. Interestingly, sex-specific differences have been reported in mitochondrial fission/fusion balance of cultured mouse cortical astrocytes, with female astrocytes having a higher level of fusion and therefore increased cell viability, an effect described to be mainly mediated by oestrogen (Arnold et al. 2008). Although astrocytic mitochondria were not measured in the present study, these findings suggest that a similar beneficial effect might happen in mitochondria of female BECs and pericytes and, when reproductive senescence strikes and sex steroid hormones decrease, the mitochondrial component may promote cell viability in pericytes but not in BECs.

BECs undergo further ultrastructural changes during ageing, especially on their surface. In the present study, BEC luminal surface projections or pseudopods, were observed to vary in size and number. Due to the limited view of the 2D analysis, this measurement was only performed in the 3D reconstructed vessels. Accordingly, pseudopods were observed in both cortex and hippocampus of young and aged female mice, but number and size of these projections were only significantly increased in aged cortical capillaries compared to young cortical capillaries. In addition, aged pseudopods were significantly higher in number and volume in cortex compared to hippocampus. The presence of pseudopods in the ageing cerebral

microvasculature is consistent with early reports of large luminal projections on the BEC surface described in senescence accelerated mice, however, the same study reported them in hippocampal vessels (E. Y. Lee et al. 2000), which is not consistent with the lack of significant hippocampal differences in our studies. Interestingly, pseudopod-like microstructures have been described in BECs under inflammatory conditions in injured mouse BBB during encephalomyelitis (Lossinsky et al. 1991), and in cultured human microvessel endothelial cells when stimulated with chemokines (CXCL10, CCL5) (Whittall et al. 2013). Also, recent studies in human endothelial cells *in vitro*, stimulated with TNF- α and interferon- γ (INF- γ), have shown surface structures that can be compared to the pseudopods observed in the present study, and also express and present adhesion molecules and chemokines (Øynebråten et al. 2015). Together, these findings suggest an increase in BEC surface in the ageing female BBB in cortex, but not in hippocampus, forming pseudopod-like structures that might mediate interaction with immune cells under pro-inflammatory conditions. In fact, a higher pro-inflammatory state in the female BBB might be supported by previous studies on differential expression patterns in the human ageing brain that showed sex dimorphic differences, with the aged female brain being more immune activated than the aged male brain (Berchtold et al. 2008).

Intercorrelation between the different cellular components of the BBB, BECs, pericytes and astrocytes, is essential for its function. BEC and pericyte interaction is enhanced in the so-called peg-and-socket interdigitations, where gap and adherens junctions allow close interconnection and passage of nutrients, metabolites or ions between both cell types (F. Li et al. 2011; S. Liu et al. 2012; Winkler, Bell, and Zlokovic 2011). Pericytes play an important role in vessel stabilization, BEC proliferation and capillary blood flow modulation, as they tend to cover BEC abluminal surface and share the same BM (Banerjee and Bhat 2007; Hellström et al. 1999), which allows endothelial-pericyte crosstalk by activating factors such as PDGF or VEGF (Darland et al. 2003; Ribatti, Nico, and Crivellato 2011). In addition, several studies have shown that BEC – pericyte contact induces TJ protein expression and therefore promotes BBB formation, integrity and maintenance (Dohgu et al. 2005; Shimizu et al. 2008). Accordingly, pericyte loss or reduced coverage is associated with vessel instability, BBB disruption and increased permeability in rodents and humans (Armulik et al. 2010; García-Quintans et al. 2016; Winkler et al. 2013). BECs and pericytes also interact with astrocytes, which mediates attachment of the astrocytic end-feet to the neurovascular unit, promoting a virtually complete ensheathment of the vessel (Armulik et al. 2010; Mathiisen et al. 2010).

Astrocytes have also been proved to promote BBB formation and maintenance in human and rodents by inducing expression of TJ proteins (claudin-5, ZO-1), redistribution of AJ protein PECAM-1 and polarized distribution of barrier transporters such as Pgp or GLUT1 (Abbott et al. 2006; Al Ahmad et al. 2011; Obermeier et al. 2013). Additionally, some studies have shown how coverage of astrocytes over BECs promotes a tighter barrier in BBB *in vitro* models of porcine, human and rat cells (Alvarez et al. 2011; Malina, Cooper, and Teichberg 2009). Ageing has been reported to promote loss of pericytic and astrocytic contacts with the vessel wall in male mice and rats, which consequently promotes BBB breakdown and neurovascular dysfunction (Duncombe et al. 2017; Hughes et al. 2006). In the current study, 3D reconstructed aged cortical capillaries showed an increase of BEC – pericyte contact in comparison to young cortical capillaries. Also, no changes were observed in hippocampal capillaries of any age. Astrocyte coverage over the vessel wall was shown to be higher in aged cortical capillaries in the 2D analysis but did not change with ageing in any of the regions analysed in the 3D reconstruction. These results are not in agreement with previous reports that showed a decrease in pericyte coverage in aged male (Bell et al. 2010) or female mice (Soto et al. 2015), or with recent studies that proved no change at all in pericyte coverage at the ageing BBB of male mice (Goodall et al. 2018) and male rats (Bors et al. 2018). Although more experiments are needed to determine the factors that contribute to these discrepancies, they may relate to technical differences between the analyses carried out using 2D images in previous experiments and the current 3D analysis, in addition to some difficulties in measuring pericyte projections. Nonetheless, as pericyte try to maximize their coverage over the endothelium during development (Berthiaume et al. 2018), perhaps something similar is happening during ageing, depending on the brain region. Together with the findings described above by Gomes et al. (2011) regarding a potential beneficial role of age-mediated changes in pericytic mitochondrial shape, these observations might suggest a compensatory mechanism in pericytes. Similarly, despite previous studies reporting a decreased astrocytic coverage over brain microvessels in male mice (Duncombe et al. 2017), our results suggest that either increase or no change in astrocytic coverage happens in the female BBB in cortex or hippocampus. Interestingly, astrocytic number has been reported to be higher in aged female mice compared to young female mice and aged male mice (Mouton et al. 2002), suggesting potential sex differences in both baseline astrocyte density and their response to age and changes in sex hormone concentrations. Indeed, sex-dependent increase in astrocyte number, and abovementioned astrocyte viability due to higher levels of mitochondrial fusion (Arnold et al. 2008), might attenuate the ageing effect on astrocytic end-feet coverage in the female BBB.

Age-induced alterations in TJ have been previously described as a key event in cerebrovascular ageing and associated BBB dysfunction in male rodents (Goodall et al. 2018; Mooradian et al. 2003). In the present study, we aimed to assess TJ morphology by measuring both tortuosity and complexity (understood as length). TJ tortuosity was significantly higher in cortical capillaries of aged female mice compared to young cortical capillaries and aged hippocampal capillaries. By contrast, TJ complexity did not show any difference due to age in cortex or hippocampus, whereas regional comparisons showed that aged cortical capillaries had a lower TJ complexity compared to aged hippocampal capillaries. However, although significant, the interregional change in TJ complexity was very small and probably has little biological meaning. Nonetheless, reduced TJ tortuosity and complexity (length) have been linked to BBB disruption in granulin mutant male mice after ischemic stroke (Jackman et al. 2013), which is not consistent with our observations in the female BBB in both cortex and hippocampus if considering the age-related BBB disruption. Also, changes in TJ morphology in blood vessels of human glioblastoma have been correlated with abnormal TJ expression and distribution (Liebner, Fischmann, et al. 2000). Therefore, if low TJ tortuosity means detrimental BBB function, the extent to which a higher TJ tortuosity is related to female BBB function according to our data is unclear. In relation, studies in middle-aged ovariectomised female rats have shown selective age-mediated loss of TJ proteins depending on the brain region, which leads to altered paracellular permeability in hippocampus (Bake et al. 2009; Bake and Sohrabji 2004), but not in cortex (Sandoval and Witt 2011), in the absence of pathology, and also in stroke. In the three studies, oestrogen activated form, estradiol, appeared to play a role in the regulation of BBB permeability. Altogether, these observations suggest that both tight junction arrangement and morphology are differentially regulated between brain regions, which may be related to loss of sex steroid hormones during reproductive senescence. Thus, further studies involving immunostaining and expression levels of specific TJ proteins would help to understand what is happening at the molecular level of these structures and if their morphological arrangement has a deeper effect on the female BBB function in ageing.

In summary, the results obtained from the ultrastructural analysis, together with findings described in literature, suggest that the ageing female BBB shows certain differences respect to the male BBB when compared to young female BBB, particularly regarding potential beneficial regulation of mitochondrial fusion/fission, a more enhanced pro-inflammatory state, age-mediated increase in pericyte coverage, unchanged astrocyte coverage and more tortuous TJs. However, sex-specific changes at the ageing BBB and how these may affect

cerebrovascular function still need further attention and research. In addition to sex differences, the present study showed that cortical brain capillaries present more changes by age than those in hippocampus. These interregional differences observed in our analysis are not consistent with previous studies that have reported the hippocampus to be equally sensitive to ageing and BBB dysfunction (E. Y. Lee et al. 2000; Lourenço et al. 2018; Topple et al. 1991), however, a recent study in aged male mice has shown cortical blood vessels to be more strongly affected by cholinergic denervation than those in hippocampus (Nizari et al. 2019), which might have important consequences in age-related vasculopathies including BBB disruption. Based on these results, posterior molecular and sequencing analyses were focused on cortical capillaries.

Chapter 3. Massive Analysis of cDNA Ends sequencing (MACE-Seq) of cortical microvessels isolated from young and aged female mice.

3.1 Introduction

Transcriptomics are used to understand molecular processes that underline cell function, including modifications at DNA and RNA levels. Most recent approaches are next generation sequencing (NGS), parallel sequencing methods that allow for effective assessment of a large set of genomic targets and at the same time provide high accuracy and sensitivity mainly due to a high sequencing coverage (Gagan and Van Allen 2015; Sahm et al. 2016). In the last few years there has been an increasing development of NGS protocols and platforms, including tools for RNA analysis at massive scale via sequencing of cDNA (RNA-Seq). The advantages of these new techniques involve detection of unannotated exons, measurement of general and exon-specific expression levels and improved detection of alternative splicing (Ozsolak and Milos 2011; Pickrell et al. 2010). Among the emerging NGS techniques, new methods have been developed including Massive Analysis of cDNA Ends (MACE-Seq), which is based on the technique of gene expression profiling by massive parallel sequencing introduced by Torres and colleagues (Torres et al. 2008), and has recently been patented by GenXPro GmbH © (Frankfurt, Germany). MACE-Seq uses a 3'-end targeted, tag-based transcriptome profiling method that produces a reliable, unbiased and accurate quantification of polyadenylated transcripts (1 transcript – 1 molecule), at a lower sequencing depth than that of standard RNA-Seq. MACE-Seq does not need to perform rRNA depletion, which allows the use of small amounts of starting material. It analyses gene expression based on high-throughput sequencing and generates a single sequence from the 3'-end of each polyadenylated transcript (Zhernakov et al. 2019a). Each cDNA molecule is represented by one cDNA fragment (tag or read) of 94 bp of size. The reads are originated from a region of 100-500 bp from the 3' (poly-A) end of the transcript. The posterior high-throughput sequencing of reads has advantages over microarray and other types of RNA-Seq in that it provides high resolution gene expression analysis of differentially expressed low-abundant transcripts. In addition, a key feature of MACE-Seq is the application of the TrueQuant (unique molecular identifiers) method for the elimination of PCR copies which ensures correct quantification of the low-complexity sequences (Zajac et al. 2015; Zawada et al. 2014). In contrast, sequencing of smaller RNA molecules (e.g. miRNA) cannot be performed using regular RNA-seq library preparation techniques nor following MACE-Seq protocol, due to their relatively small size.

Therefore, in this study Small RNA analysis was used to characterize non-coding small RNAs, focusing in miRNAs. Small RNA-seq library preparation is usually performed by ligation of adapters to the RNA molecules followed by reverse transcription and PCR amplification. Similarly to MACE-Seq, TrueQuant method was used for elimination of PCR copies and ensuring correct quantification (Müller et al. 2015). The most recent kits for library preparation avoid generation of adapter-adapter products and allow the use of even smaller amounts of starting material (Lipps et al. 2019).

Ageing promotes accumulation of molecular modifications that affect several tissues including the brain (Lee, Weindruch, and Prolla 2000). In females, the ageing process is highly influenced by the effect of steroid sex hormones, which are important for brain function (Moraga-Amaro et al. 2018) and gene expression modulation (Rinn and Snyder 2005). Progesterone and oestrogen have several roles in neuroprotection (Berent-Spillson et al. 2015; Gibson, Coomber, and Murphy 2011; Jiang et al. 2016). Similarly, oestrogen has been linked to cerebrovascular protection by promoting mitochondrial efficiency and reducing oxidative stress (Stirone et al. 2005). Also, oestrogen has been reported to reduce expression of proinflammatory cytokines, adhesion molecule expression and leukocyte transmigration in the brain vasculature (Corcoran et al. 2010; Maggioli et al. 2016). In addition, progesterone and oestrogen appear to modulate circulating antioxidant enzymes (Bellanti et al. 2013). Therefore, changes in sex hormone levels that happen during and after menopause might have an effect in some brain functions in aged women and female rodents. Indeed, oestrogen decline leads to loss of BBB integrity in animal models (Wilson et al. 2008). Moreover, reduced circulating oestrogen levels appear to have a negative effect on amyloid- β clearance (Li et al. 2000). Several miRNAs are also differentially expressed in the ageing mouse brain in comparison to young mice (Goodall et al. 2019; Inukai et al. 2012; N. Li et al. 2011). Indeed, miRNAs have been suggested as useful ageing biomarkers and they also contribute to the modulation of several processes including neuroinflammation, response to DNA damage or cellular senescence (Lal et al. 2009; X. Li et al. 2011; Olivieri, Lazzarini, et al. 2013). Furthermore, miRNA role in cerebrovascular dysfunction has also been highlighted. In fact, previous studies have reported miRNA contributing to regulation of TJ protein expression as well as BEC survival, thus affecting BBB permeability and integrity (Fang et al. 2016). Similarly, previous studies have shown how miRNAs such as miR-155 promote increased leukocyte adhesion on brain endothelium during neuroinflammation (Cerutti et al. 2016). Conversely, other miRNAs such as miR-125a promote decreased expression of adhesion molecules and leukocyte adhesion

on brain endothelium (Reijerkerk et al. 2013). Thus, the balance of miRNA expression is also essential for both brain and BBB function. All these observations suggest that age-induced molecular alterations in the female brain might be related to hormonal changes and may contribute to impaired stress response, neuroinflammation and BBB dysfunction, by modulating changes in both mRNA and miRNA expression.

The aim of this chapter was to perform MACE-Seq and Small RNA analysis (GenXPro GmbH ©, Frankfurt, Germany) on RNA samples of brain microvessels from young and aged C57BL/6J female mice, in order to assess age-related changes in gene and miRNA expression at the female BBB. Potential molecular changes at gene expression could explain functional and structural changes observed at the ageing BBB. After sequencing analyses were performed, miRNA target prediction tools were used in order to select an age-deregulated pair of miRNA/target mRNA for further analysis regarding their putative role in cell function and BBB properties.

3.2 Material and Methods

3.2.1 *Animals*

All animal work and welfare were performed as described in Chapter 2 of this thesis.

3.2.2 *Microvessel Isolation protocol*

3.2.2.1 *Brain collection and processing of the cortex*

For this protocol, young and aged C57BL/6J female mice were perfused intracardially using 0.5% bovine serum albumin (BSA) solution (BSA fraction V, 25% (#05482, Sigma-Aldrich, Dorset, UK), HEPES, 10 mM (#H0887, Sigma-Aldrich, Dorset, UK), antibiotics Penicillin/Streptomycin, 100 U/ml (#15140-122, ThermoFisher Scientific, Loughborough, UK) in DMEM (#21063045, ThermoFisher Scientific, Loughborough, UK). After perfusion, brains were carefully extracted and stored in ice-cold 0.5% BSA solution until the left and right cortices were dissected. Cortices were then rolled on a dry filter paper to remove the meninges. Dissected cortices of 5 mice were pooled together in a same tube for each sample in each age

group (n=3 pooled samples/group). An initial trituration was performed using a sterile scalpel to secure an efficient digestion in the following steps.

3.2.2.2 *BEC isolation protocol testing – Magnetic bead isolation*

To determine the isolation method that yielded the purest and more concentrated fraction of BECs for transcriptomic analysis, 7 different protocols were tested (n=1 mouse brain per protocol). These initial protocols used a combination of double enzymatic digestion, filtration with different mesh sizes and use of puromycin or magnetic beads to increase the presence of BECs in the suspension. Only the magnetic bead-mediated isolation protocol was replicated 3 times. For single BECs isolation by magnetic beads, source tissue was digested by a solution of collagenase/dispase (collagenase/dispase (#10269638001, Sigma-Aldrich, Dorset, UK), 1mg/ml, DNase I (#DN25, Sigma-Aldrich, Dorset, UK), 10 mg/ml; N-Tosyl-L-lysine chloromethyl ketone (TLCK), 147 µg/ml (#T7254, Sigma-Aldrich, Dorset, UK)) in DMEM for 1h at 37 °C with occasional vigorous shaking. Glass Pasteur pipettes were used to homogenize the suspension until reaching a creamy texture. The suspension was then centrifuged at 700g for 5 min, supernatant was removed, and the pellet was resuspended in 20 ml of 25% BSA solution in DMEM. Centrifugation (1000g, 20min) was performed to create a BSA gradient that dragged microvessel fragments to the bottom of the tube, whereas the rest of contaminants and debris formed a plug-in at the top. The plug-in and BSA were transferred to another tube, microvessel fragments retained at the bottom were resuspended in 0.5% BSA solution and this procedure was repeated three times. A second enzymatic digestion was performed using porcine trypsin-EDTA 0.25% (w/v) (#11570626, ThermoFisher Scientific, Loughborough, UK) at 37°C for 5 min, and suspension was then filtered (40 µm cell strainer (#10737821, ThermoFisher Scientific, Loughborough, UK)). Finally, positive separation was performed using anti-CD31 coated magnetic beads and columns from Miltenyi Technologies (#130-097-418 and #130-042-201, Miltenyi Technologies, Bergisch Gladbach, Germany). CD31-positive cells were retained in the column, then flushed out and collected into fresh RNase-free tubes. Three experimental replicates were performed, samples from 5 animals per age group were pooled together in each independent repeat (n=3/age group), centrifuged at 300g for 10 min and then resuspended in lysis buffer for RNA extraction and stored at -80°C. Relative expression values were calculated as the expression in the isolated fraction referred to the whole cortex as control.

3.2.2.3 *Microvessel fragments isolation protocol*

The protocol to isolate microvessel fragments was a variation of the one described above for BEC isolation with magnetic beads. Following the initial trituration, the suspension was incubated in the same enzymatic collagenase/dispase solution for 1h at 37 °C, with occasional vigorous shaking. Glass Pasteur pipettes were used to homogenize the suspension, which was then centrifuged at 700g for 5min, supernatant was removed, and the pellet was resuspended in 20 ml of 25% BSA solution in DMEM. Microvessel fragments were retained at the bottom of the tube, following the same BSA gradient as the one mentioned above for the magnetic bead protocol. Suspension was secondly digested using trypsin-EDTA 0.25% at 37°C for 3 min. Finally, filtration using cell strainers of 40 µm was performed. Three independent experimental repeats were performed, and all microvessel fragments from 5 mice per age group were pooled together in each repeat (n=3/age group). Microvessels were then centrifuged at 300g for 10 min, resuspended in lysis buffer for RNA extraction and stored at -80°C. Again, relative expression values were calculated as the expression in the isolated fraction referred to the whole cortex as control. The RNA extracted from these samples was used for both MACE-Seq and relative expression analyses.

3.2.3 *RNA isolation*

For the testing, total RNA from BECs and microvessel fragments was extracted using a commercial kit, RNeasy Plus Mini (50) (#74134, Qiagen, Manchester, UK) according to manufacturer's instructions. For the samples used in the RNA-Seq, total RNA was extracted using miRCURY RNA isolation kit (#300112, Exiqon, Vedbaek, Denmark) following the protocol provided by the manufacturer. In every case, final RNA samples were stored in the freezer at -80 °C until further analysis.

3.2.4 *RNA-sequencing: Massive Analysis of cDNA Ends (MACE-Seq)*

3.2.4.1 *Generation of MACE-Seq libraries and RNA sequencing*

A quantitative genome-wide expression profiling was performed with RNA extracted from microvessel fragments of 6 and 24-month-old female mice. This analysis was performed in collaboration with Ricardo Figueiredo and Peter Winter from GenXPro GmbH. MACE-Seq

technique follows a modified protocol described in Nold-Petry et al. (Nold-Petry et al. 2015) and was performed at GenXPro GmbH. Samples of 100 ng of DNase-treated RNA were used for library preparation. A total of 27 MACE-Seq libraries were generated using the MACE-Seq kit v2.0 (GenXPro GmbH, Frankfurt-Am-Main, Germany) according to the supplier's protocol by the researchers 'blinded' to the identity of the samples. Reverse transcription was performed to synthesise cDNA molecules using oligo primers, which were fragmented to an average size of 200 bp by sonication using a bioruptor (Diagenode, Seraing, Belgium). Total DNA was quantified using Qubit HS dsDNA assay (#Q33230, ThermoFisher Scientific, Loughborough, UK). Afterwards, cDNA fragments were ligated to DNA adapters containing TrueQuant unique molecular identifiers included in the MACE-Seq kit v2.0. For library amplification, PCR was used following purification by solid phase reversible immobilization beads Agencourt AMPure XP (#A63881, Beckman Coulter, Brea, CA, USA). Lastly, sequencing was performed using a NextSeq Illumina platform (Illumina Inc., San Diego, CA, USA). For transcript quantification and abundance comparison between samples, sequencing reads were normalized to the mean of each replicate.

3.2.4.2 Bioinformatic analysis

Around 6 million MACE-Seq reads were obtained from all the libraries. PCR-duplicates were removed from raw data after identifying them using the aforementioned TrueQuant identifiers. After eliminating the PCR duplicates, the remaining reads were poly(A)-trimmed and the low-quality ones were also removed. After this treatment, the resultant reads were aligned to the mouse reference genome (*Mus musculus*, GRCm38. p2, Ensembl release 74, December 2013) using bowtie2 mapping tool. As a result, a dataset with a total of 28552 identified genes was obtained. The gene count data was normalized to account for differences in sequencing depth. Differential gene expression was assessed using the statistical programming software R (www.rproject.org). DeSeq2 R/Bioconductor package was used to estimate fold change and calculate p -values, as described previously (Love, Huber, and Anders 2014). This resulted in a p -value and log₂-fold change (log₂FC) for every gene sequenced. False discovery rate (FDR) analysis was estimated to account for multiple testing. Genes with a p -value lower than 0.05 and a log₂FC equal to or higher than [1] were considered differentially expressed. These differentially expressed genes were later categorized into upregulated or downregulated genes. Up- and downregulated genes were further assigned to

biological pathways to analyse age-mediated alterations by gene set enrichment analysis using DAVID Bioinformatics.

3.2.4.3 *Quantification of microRNA expression*

Ultra-deep miRNA sequencing was performed from pooled small RNA from young and aged mice, to assess whether changes in gene expression could be related to deregulation of miRNA expression in ageing. Total RNA, including small RNA, was extracted using miRCURY RNA isolation kit from Exiqon as mentioned above. The following protocol was performed at GenXPro GmbH in collaboration with Ricardo Figueiredo and Peter Winter. Small RNA libraries were prepared using TrueQuant technology for elimination of PCR bias. Briefly, 3' and 5' adapters were ligated onto small RNA (<200 nucleotides) using T4 RNA Ligase 2 and T4 RNA Ligase 1 (#M0239S and #M0204S, New England Biolabs, Frankfurt-Am-Main, Germany) respectively. Reverse transcription of adapter-ligated RNA was performed with SuperScript III reverse transcriptase (#18080044, ThermoFisher Scientific, Loughborough, UK) and amplified by PCR with KAPA HiFi Hot-Start Polymerase (#KK2602, KAPA Biosystems, Roche; Darmstadt, Germany). Afterwards, amplified libraries were sequenced using Illumina sequencing platform (Illumina Inc., San Diego, CA, USA). Analysis of Small RNA-seq data was performed using omiRas mapped to the mouse genome by Bowtie2 tool. Differentially expressed mature miRNAs between young and aged samples showed a *p*-value lower than 0.05 and a log₂FC equal to or higher than [1].

3.2.4.4 *MiRNA target prediction analysis*

Targets for the significantly deregulated miRNAs were selected from the list of deregulated genes sequenced in the MACE-Seq analysis. Two target prediction tools were used in combination, TargetScan7.2 and miRWalk2.0, to narrow down the list of genes based on their identification as miRNA targets in at least 6 out of 11 databases. The list of targets was reduced by excluding target genes where the pattern of expression (e.g. increased or decreased) was the same between the mRNA and predicted miRNA regulators.

3.2.5 Reverse transcription – Quantitative PCR

Reverse Transcription-Quantitative PCR (RT-qPCR) analysis was performed as a way of measuring the purity of the isolated fractions as well as to validate the age-related changes in expression observed in the MACE-Seq results. Distinct cell markers were used for the preliminary purity assessment, including PECAM-1 (or CD-31) for BECs (*Mus musculus*, F:5'-GGACGATGCGATGGTGTATAA-3'; R: 5'-GCATCACTGTGCATTTGTA CTT-3'), glial fibrillary acidic protein (GFAP) for astrocytes (*Mus musculus*, F:5'-CAGAGGAGTGGTATCGGTCTAA-3'; R: 5'-GATAGTCGTTAGCTTCGTGCTT-3'), SMA- α for smooth muscle cells (*Mus musculus*, F:5'-TCAGGGAGTAATGGTTGGAATG-3'; R: 5'-GGTGATGATGCCGTGTTCTA-3'), PDGF β receptor (PDGFR- β) for pericytes (*Mus musculus*, F:5'-GACAGACATGATGGACAGTGAG-3'; R: 5'-TGGCAGTTGAGGTGGTAATC-3') and actin- β as a reference gene (*Mus musculus*, F:5'-CTCCCTGGAGAAGAGCTATGA-3'; R: 5'-CCAAGAAGGAAGGCTGGAAA-3'). This technique was also used to validate the expression changes observed on the RNA-seq data for several genes, including Bambi (*Mus musculus*, F:5'-ACTCCAGCTACTTCTTCATC-3'; R: 5'-TAGCATCTGATCTCTCCTTTG-3'), Ccl5 (*Mus musculus*, F:5'-AGGAGTATTTCTACACCAGC-3'; R: 5'-CAGGGTCAGAATCAAGAAAC-3'), Mmp9 (*Mus musculus*, F:5'-GTTTTCTTCTTCTCTGGACG-3'; R: 5'-CTAGACCCA A CTTATCCAGAC-3'), Tirap (*Mus musculus*, F:5'-GTTATACACTATCTGGAGACAC-3'; R: 5'-GATCTGATCCTGTGTCATAAAC-3'), Tle1 (*Mus musculus*, F:5'-GAAGGTGGATGATAAGGATAAC-3'; R: 5'-CAGACGGTTTTTGTCAATTC-3'), Mapk7 (*Mus musculus*, F:5'-AGATCTGTCTATGTGGTACTG-3'; R: 5'-CTGGTACAGGAAGTATCTCAC-3') and Dnmt3a (*Mus musculus*, F:5'-T TACTTCTGGGGTAACCTTC-3'; R: 5'-CTTTATAGAGTTTGACCTGGTG-3'). All primers used were pure and simple primers obtained from Sigma-Aldrich (#VC00026, Sigma-Aldrich, Dorset, UK). For mRNA, two commercial kits were used to perform RT-qPCR, High Capacity Reverse Transcription Kit (#4368814, ThermoFisher Scientific, Loughborough, UK), to obtain cDNA from the RNA samples, and QuantiTect SYBR Green PCR Kit (#204141, Qiagen, Manchester, UK) to perform the qPCR itself. For miRNAs, TaqMan Expression Advanced Assays miRNA cDNA synthesis kit, TaqMan Fast Advanced Master Mix plus specific probes (#A28007, #4444557 and #A25576, ThermoFisher Scientific, Loughborough, UK) were used. All the protocols used in this section were provided by the manufacturer.

3.2.6 *Relative expression and tags per million normalization*

For RT-qPCR analysis, expression of each marker relative to the control was calculated using the $\Delta C(t)$ method previously described (Tai et al. 2010). The samples from young mice were considered as control in this study. The reference gene used to measure relative expression of mRNA was actin- β and miRNA-24-3p was used as the reference for relative expression of miRNAs.

Tags (or reads) per million (TPM) is a normalization method for sequencing results and it was used to represent normalized expression levels of selected genes and miRNAs from MACE-Seq and Small RNA analysis. This method is understood as the number of RNA molecules that come from a certain gene/transcript for every 1,000,000 RNA molecules in the sequencing sample.

3.2.7 *Immunofluorescence staining*

Frozen brains from young and aged mice (n=4/group) were sectioned by cryostat at 20 μ m thickness, collected onto slides and stored at -20 °C for further analysis. Sections were post-fixed by incubation in acetone for 10 min on ice. Sections were washed with 0.01M PBS (3 times, 5 min), then incubated with citrate buffer (0.01 M Sodium Citrate in 0.01% tween pH 6) (Sigma-Aldrich, Dorset, UK) at ~90 °C (3 times, 5 min) in order to perform antigen retrieval. Afterwards, sections were blocked with 15% goat serum (ThermoFisher Scientific, Loughborough, UK) in 0.01M PBS for 15 min and then incubated overnight at 4 °C with anti-DNMT3A (1:200, #MA5-16171, ThermoFisher Scientific, Loughborough, UK) in 0.01M PBS. The next day, sections were washed with 0.01M PBS (3 times, 5 min) and incubated with AlexaFluor488 anti-rabbit (1:200, #A32731, ThermoFisher Scientific, Loughborough, UK) in 0.01M PBS for 2h at RT, protected from light. Slides were coverslipped using Mowiol ® (Sigma-Aldrich, Dorset, UK) containing 0.1% v/v Citifluor (Citifluor Ltd, London, UK) as mounting media. Slides were left in the fridge to dry and sections were later imaged using confocal microscopy Leica confocal N1057 (Leica Microsystems Ltd, Milton Keynes, UK). To assess immunofluorescence staining of Dnmt3a in mouse brain tissue, 4 animals per age group were used (n=4/ age group), with 3 replicates each.

3.2.8 *Statistical analysis*

In the case of MACE-Seq and pathway analysis, DeSeq2 R/Bioconductor and Fisher's Exact Test were used respectively. For the rest of analyses, GraphPad Software (Prism 8.2.0, La Jolla, USA) was used. Shapiro-Wilk normality test and Q-Q plots were used to assess distribution of the data. Data from purity and cell marker expression experiments was analysed using one-way ANOVA and Dunnett's correction test. Unpaired two-tailed Student's t test was used to analyse qPCR validation and immunofluorescence analyses. Significance was set at $p < 0.05$ and results are presented as mean \pm SEM. The selection of appropriate statistical tests is indicated in each figure legend.

3.3 Results

3.3.1 *Microbead-mediated isolation generates high purity of brain endothelial cells but low RNA yield*

To determine the isolation method that generated the best BEC purity and yield, different isolation methods including density centrifugation, addition of puromycin and immunopanning with magnetic beads were tested on mouse cortices (data not shown). Among these, tissue processing using anti-CD31-coated microbeads yielded fractions that were significantly enriched in BECs. Other BBB markers were also present in the fraction but no significant differences were observed in comparison to the whole cortex (**Figure 23**).

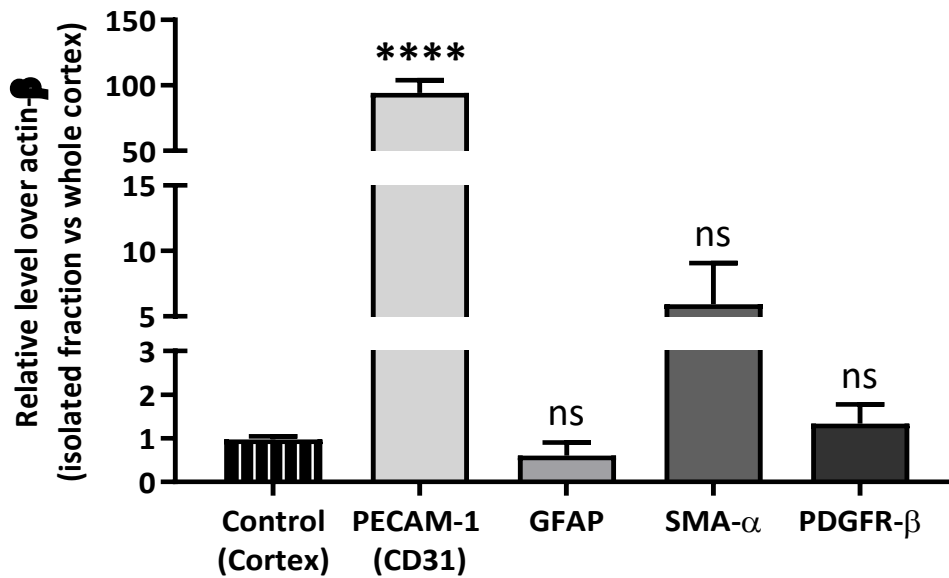


Figure 23. Relative expression of blood-brain barrier cell markers in isolated BEC fractions compared to the whole cortex as control.

The markers used were PECAM-1 (or CD-31; for BECs), GFAP (for astrocytes), SMA- α (for smooth muscle cells), PDGFR- β (for pericytes) and actin- β as the inner control gene. According to these results, the samples were significantly enriched in BECs, however, other BBB cell types were still present. (n=3, One-Way ANOVA and Dunnett's correction test; ****, $p < 0.0001$; ns, not significant).

However, the yield of total RNA obtained from BEC-enriched fractions was quite low in both young and aged mice ($0.15\mu\text{g} \pm 0.04$ for young; $0.15\mu\text{g} \pm 0.03$ for aged, per pooled cortices), and therefore unlikely to be sufficient for posterior sequencing analysis. Thus, to determine whether RNA yield could be improved, microvessel fragments were isolated from dissected cortices instead of single BECs. As shown in **Figure 24**, endothelial purity was lower than that obtained when using microbeads. Also, in addition to PECAM-1-positive BECs, smooth muscle cells and pericytes were also significantly increased in the microvessel fraction. GFAP-positive astrocytes were also present but not significantly different from cortical levels.

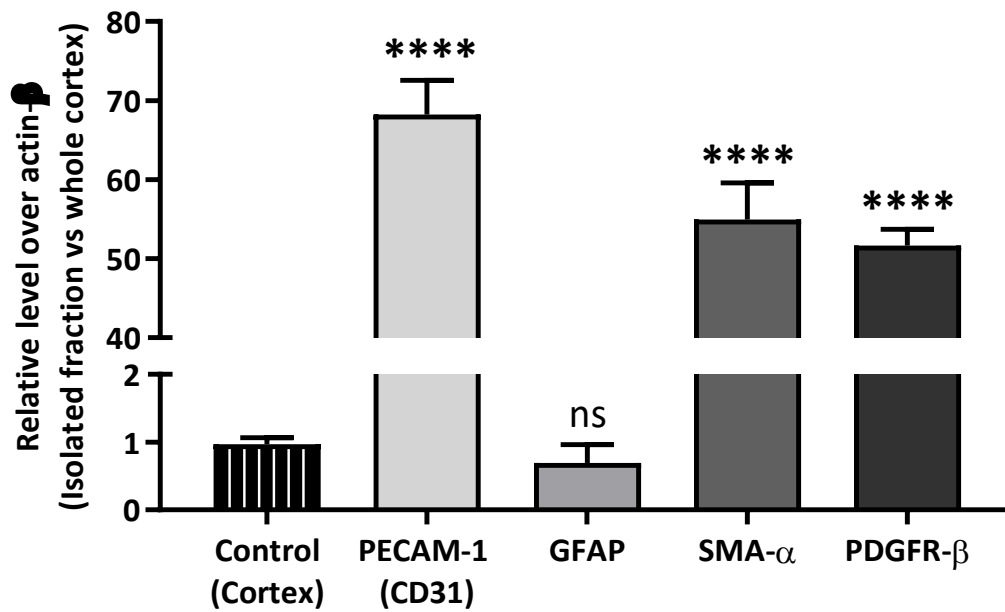


Figure 24. Relative expression of blood-brain barrier cell markers in isolated microvessel fragment fractions compared to the whole cortex as control.

The markers used were PECAM-1 (or CD-31; for BECs), GFAP (for astrocytes), SMA- α (for smooth muscle cells), PDGFR- β (for pericytes) and actin- β as the inner control gene. According to these results, the samples were significantly enriched in BECs, smooth muscle cells and PDGFR- β when compared to expression levels of their markers in the whole cortex. GFAP mRNA expression levels were found to be not significantly different between the cortex and the isolated microvessel fragments. (n=3, One-Way ANOVA and Dunnett's correction test; ****, $p < 0.0001$; ns, not significant).

Total RNA was extracted from microvessel fragments as explained before but using in this case miRCURY RNA isolation kit from Exiqon. The yield of total RNA obtained was improved compared to single BEC isolation ($1.8\mu\text{g} \pm 0.08$ for young; $1.81\mu\text{g} \pm 0.01$ for aged, per pooled cortices). This protocol ensures the amount of RNA required to perform posterior sequencing analysis even though BBB marker analysis suggests that there is presence of other cells from the BBB in the microvessel fragment fractions. Therefore, this was the protocol selected to collect the samples from young and aged female mice for MACE-Seq analysis.

To determine whether the abundance of different BBB cell types within the microvessel fraction changed with age, microvessel fractions from young and aged mice were analysed by RT-qPCR and levels of BBB marker expression in aged mice relative to young animals were calculated. No differences were noted in the relative levels of endothelial cell and astrocyte

markers between young and aged microvessel fractions. However, RNA levels of smooth muscle cells and pericytes were significantly lower in the aged samples (**Figure 25**).

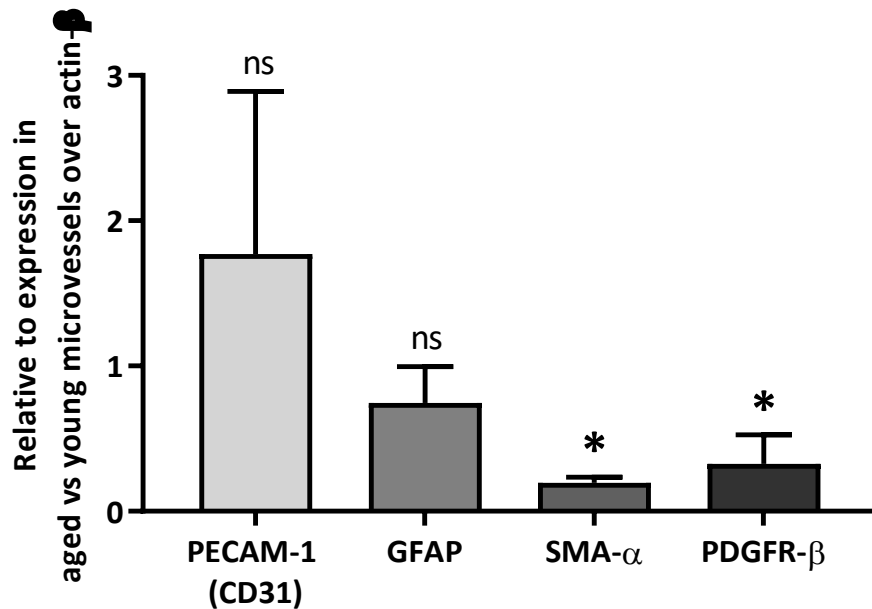


Figure 25. Relative expression of BBB cell markers in isolated aged microvessel fragments compared to young microvessels as control.

Differences in sample purity of aged versus young microvessel fragments were assessed by calculating relative expression of different BBB cell markers. The markers used were PECAM-1 (or CD-31; for BECs), GFAP (for astrocytes), SMA-α (for smooth muscle cells), PDGFR-β and Actin-β as the control gene. Young microvessel fragments were used as control, thereby changes in fold-change represent how gene expression changes in aged microvessels respect to young microvessels. According to these results, the samples are enriched in BECs although other BBB cell types are still present. Only the ratio of smooth muscle cells and pericytes seems to be significantly decreased in aged animals (n=3, One-way ANOVA and Dunnett's correction test; *, $p < 0.05$; ns, not significant).

3.3.2 Ageing promotes deregulation of inflammation, signalling and metabolic related pathways by promoting the differential expression of messenger RNAs and microRNAs in the female BBB

RNA samples extracted from the cortical microvessel fractions of young and aged female mice were analysed using MACE-Seq and small RNA analysis. Over 300 mRNAs were found to be differentially expressed between young and aged microvessels samples in the

MACE-Seq analysis (**Figure 26A**). Of these, 211 genes were upregulated and 94 were downregulated in aged animals (**Figure 26B**). Pathway analysis was then performed on DAVID bioinformatics. A total of 10 most upregulated and 10 most downregulated pathways were selected and according to the GO terms obtained, the majority of upregulated genes were associated with immune system process, cytokine production, leukocyte migration and inflammatory response related pathways (**Figure 26C**). Significantly, downregulated genes were associated with pathways related to metabolic and signalling pathways including intracellular signal transduction and MAPK cascade. Interestingly, both deregulated gene groups also showed enrichment for genes related to common GO terms such as response to stress and regulation of vascular development. A list with all differentially expressed genes can be found in the appendix of this thesis (**page 208, Table 5**).

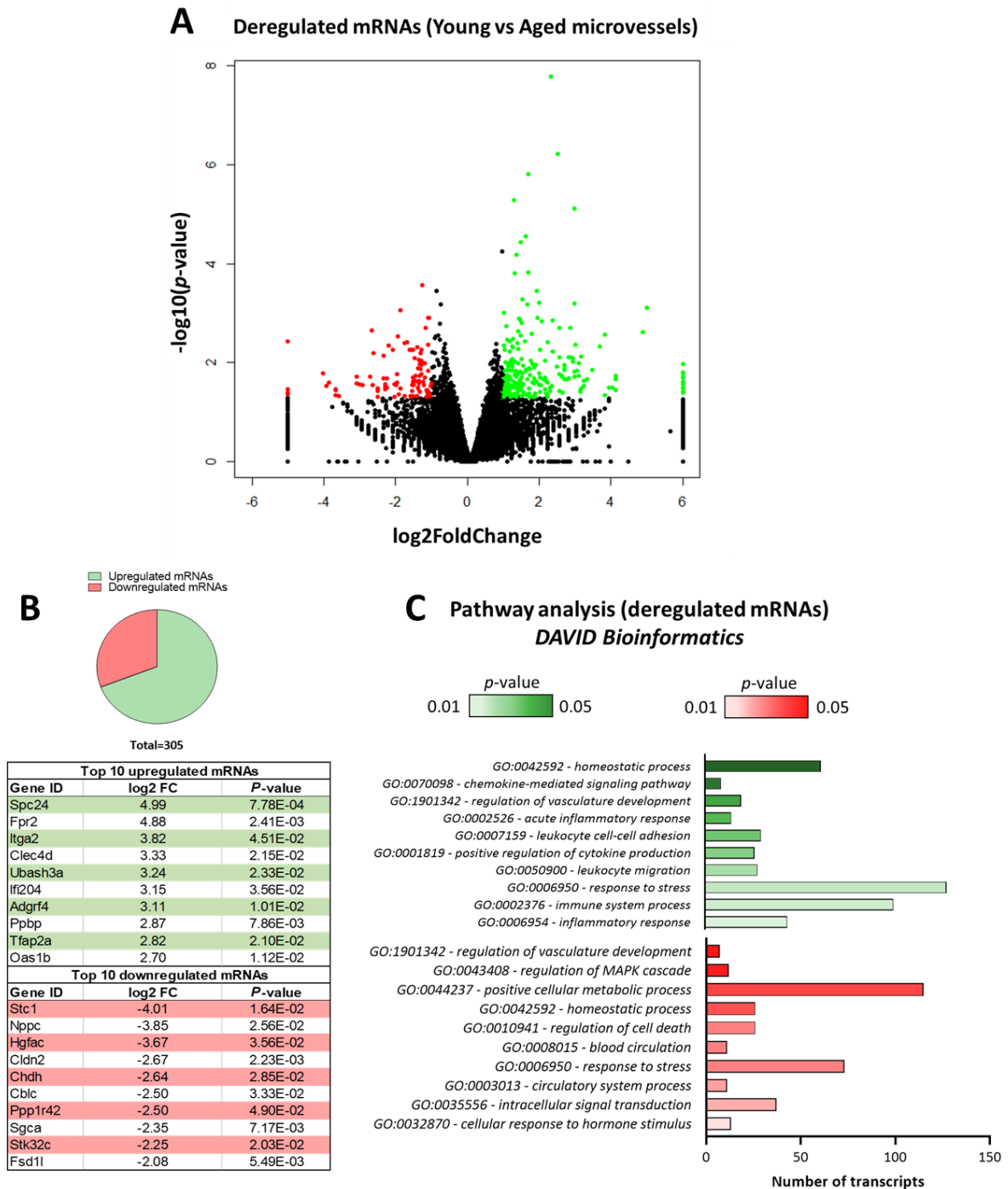


Figure 26. Upregulation (positive log₂FC) and downregulation (negative log₂FC) of mRNAs in aged female brain microvessel fragments compared to young female mice.

MACE-Seq analysis performed in brain microvessel fragments of aged and young female mice showed more than 300 differentially expressed genes (p -value<0.05; log₂FC>[1]). **A**) Volcano plot distribution of deregulated mRNAs from young and aged microvessel fragments (upregulated: green; downregulated: red). **B**) Pie plot representing the number total of deregulated mRNAs (305), among them 211 were upregulated and 94 were downregulated. Top 10 upregulated and downregulated genes are presented in the table. **C**) Pathway analysis was performed on DAVID Bioinformatics and, according to GO terms obtained, upregulated genes were mostly

associated with immune system process, cytokine production, leukocyte transigrations and inflammatory response. The majority of downregulated genes were associated with metabolism and signalling pathways (e.g. intracellular signal transduction and MAPK cascade). (n=3, Fisher Exact's Test, $p < 0.001$ to $p < 0.05$).

3.3.2.1 Age-mediated deregulation of microRNA expression in the female BBB

Analysis for small RNAs was also performed on brain microvessel fragments from young and aged female mice, in order to assess alterations in miRNA expression. This analysis showed that 9 miRNAs were differentially expressed between young and aged mice, which included 4 upregulated and 5 downregulated miRNAs (**Figure 27A-B**). Target prediction tools (TargetScan 7.2, miRWalk 2.0) were used to identify potential predicted targets of these 9 deregulated miRNAs among the list of differentially expressed mRNAs previously identified. Pathway analysis via DAVID Bioinformatics indicated that deregulated predicted targets were mostly involved in small GTPase signal transduction, cell death, angiogenesis, vascular development, cell migration or response to stress (**Figure 27C**).

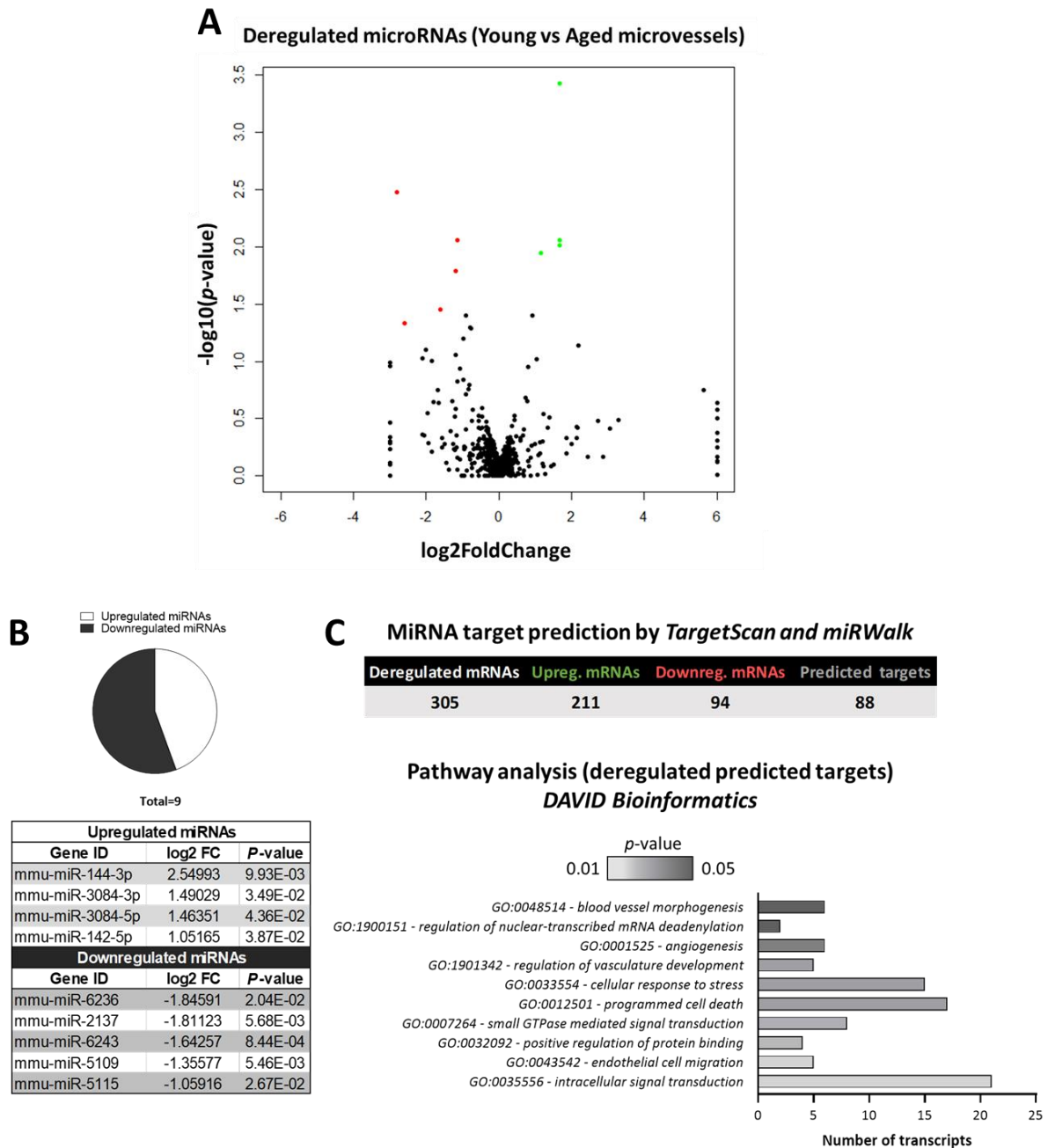


Figure 27. Age-deregulated miRNAs and predicted targets in female brain microvessel fragments.

Small RNA analysis performed in brain microvessel fragments of aged and young female mice showed 9 differentially expressed miRNAs ($p\text{-value} < 0.05$; $\log_2\text{FC} > [1]$). **A**) Volcano plot distribution of deregulated miRNAs from young and aged samples (upregulated: green; downregulated: red). **B**) Pie plot representing the total number of deregulated miRNAs (9), of which 4 were upregulated and 5 were downregulated. Both upregulated and downregulated miRNAs are presented in the table. **C**) TargetScan 7.2 and miRWalk 2.0 were used to identify predicted targets of these deregulated miRNAs. Pathway analysis was performed on DAVID Bioinformatics using predicted target mRNAs and the GO terms obtained showed that these deregulated targets were mostly associated with small GTPase signal transduction, cell death, angiogenesis, vascular development, cell migration and response to stress. ($n=3$, Fisher Exact's Test, $p < 0.001$ to $p < 0.05$).

3.3.3 The effect of age-mediated deregulation on gene expression may vary depending on the analysis used

To confirm the MACE-Seq results, age-related changes in gene expression were validated using qPCR with the same RNA samples from young and aged microvessel fragments. Genes were selected to be validated if they were found to be both significantly deregulated in the aged brain and associated with pathways involved in cerebrovascular function, according to literature. In total, 3 mRNAs that were upregulated in MACE-Seq (*Bambi*, *Ccl5* and *Mmp9*) and 4 mRNAs that were significantly decreased (*Tirap*, *Dnmt3a*, *Mapk7* and *Tle1*) were selected for validation (**Figure 28A–B**). *Bambi* did not show any differences in expression in aged microvessels compared to young mice. *Ccl5* and *Mmp9* expression levels showed a non-significant trend towards upregulation in aged animals. In contrast, *Tirap* showed a trend towards downregulation in aged mice. On the other hand, *Tle1*, *Dnmt3a* and *Mapk7* mRNA levels were significantly lower in aged microvessels compared to young animals.

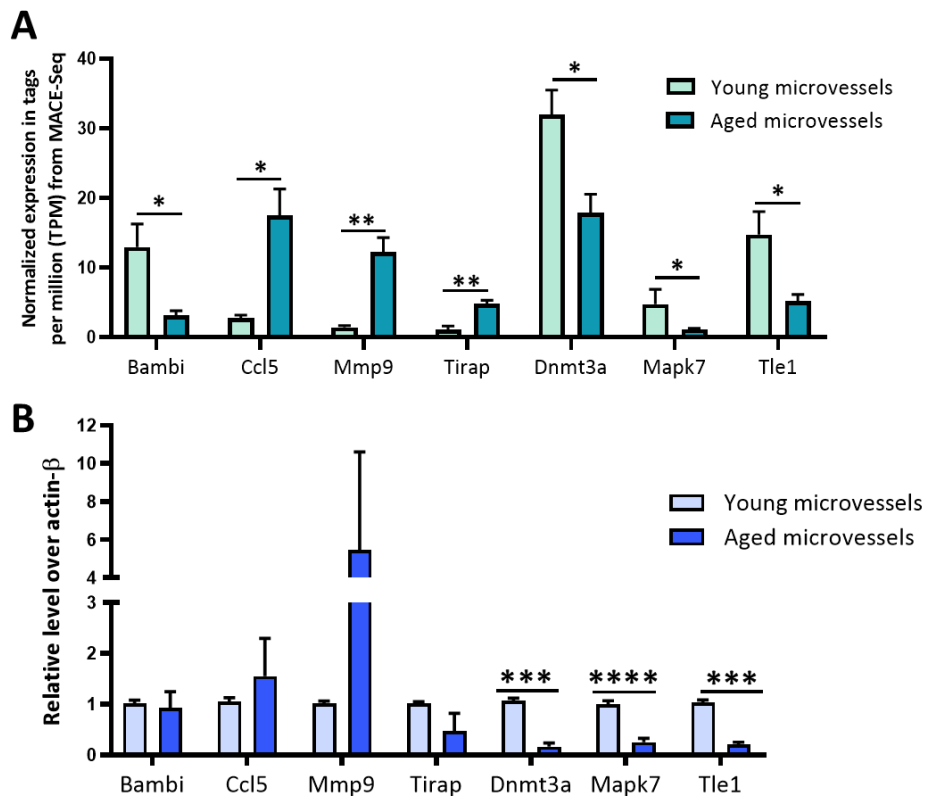


Figure 28. Validation of age-mediated mRNA deregulation by qPCR in brain microvessel fragments of aged and young female C57BL/6J mice.

A) A total of 7 mRNAs were assessed in order to validate the age-mediated deregulation in expression observed in the MACE-Seq analysis. **B)** Relative expression was measured by qPCR using actin- β as internal control. Out of 7 miRNAs tested, only 3 mRNAs (Tirap, Dnmt3a, Mapk7) showed a significant deregulation by qPCR, being downregulated in aged microvessel fragments compared to young mice. (n=3, Student's t test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Changes in expression of age-deregulated miRNAs were also validated by qPCR. Out of the original 9 miRNAs identified from the Small RNA analysis, only 7 were tested due to lack of commercial qPCR primers for miRNA-6243 and miRNA-5109. In total, 4 mRNAs that were upregulated in Small RNA analysis (miRNA-3084-5p, miRNA-3084-3p, miRNA-144-3p and miRNA-142-5p) and 3 mRNAs that were significantly decreased (miRNA-2137, miRNA-6236 and miRNA-5115) were selected for validation (**Figure 29A–B**). MiR-5115, miR-6236 and miR-2137 expression levels did not show significant differences in aged mouse microvessels compared to those of young mice. In contrast, miR-3084-5p, miR-3084-3p, miR-144-3p and miR-142-5p levels were significantly higher in aged microvessels in comparison to young mice.

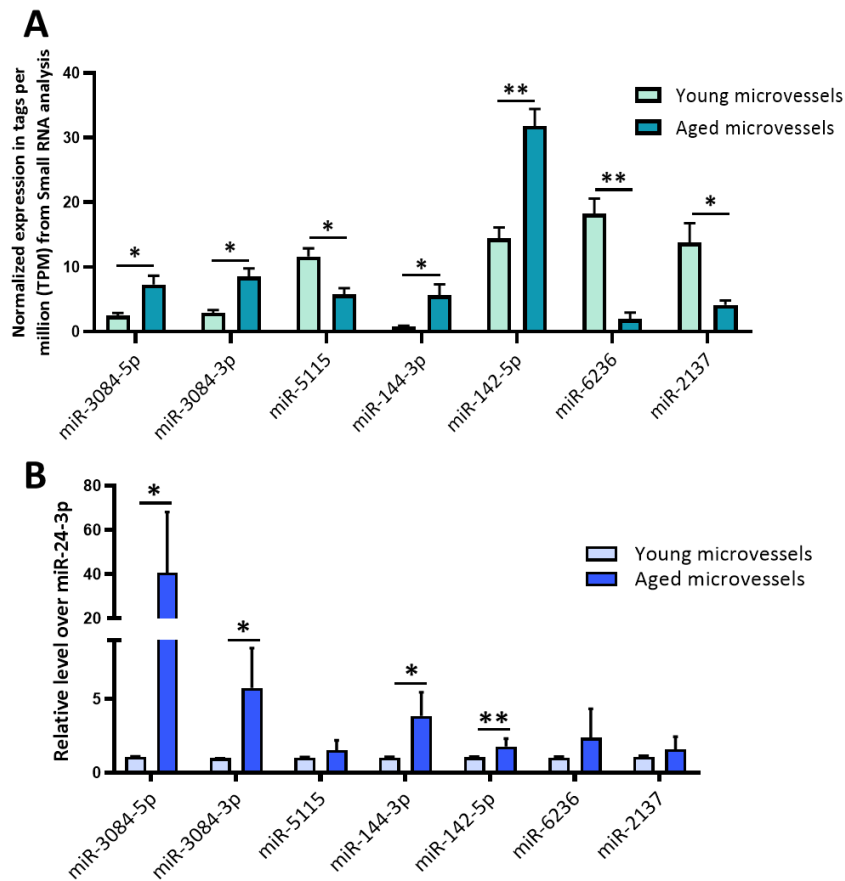


Figure 29. Validation of age-mediated microRNA deregulation by qPCR in brain microvessel fragments of aged and young female C57BL/6J mice.

A) A total of 7 miRNAs were assessed in order to validate the age-related changes in expression obtained from Small RNA sequencing analysis. **B)** Relative expression was measured by qPCR using miR-24-3p as internal control. Out of 7 miRNAs tested, only 4 (miR-3084-5p, miR-3084-3p, miR-144-3p and miR-142-5p) showed a significant deregulation, being overexpressed in aged microvessel fragments compared to young animals. (n=3, Student's t test; *, $p < 0.05$; **, $p < 0.01$).

From the differentially expressed mRNAs and miRNAs, *Dnmt3a* and miR-144-3p were of great interest due to their described role in literature. Prediction tools used in this study, TargetScan 7.2 and miRWalk 2.0, showed that *Dnmt3a* is predicted to be targeted by miR-144-3p in human and mouse, in both of which this relationship appears to be conserved (**Figure 30**).



Figure 30. Sequence alignment of *Dnmt3a*/*DNMT3A* and miR-144-3p in mouse and human by TargetScan.

A) Prediction of *Dnmt3a* as a biological target of miR-144-3p in mouse. B) *DNMT3A* is predicted to be a biological target of miR-144-3p in human. TargetScanMouse and TargetScanHuman were used to assess this prediction.

Results in both mRNAs and miRNAs presented above showed a certain degree of variability between sequencing analyses and qPCR techniques. Nonetheless, significant differences in expression levels of miR-144-3p and *Dnmt3a* due to ageing were confirmed by qPCR and sequencing results (assessed by tags per million, TPM). According to these results, *Dnmt3a* RNA levels were significantly reduced in aged mice compared to young animals

(**Figure 28**). By contrast, miR-144-3p was significantly overexpressed in aged brain microvessels compared to young microvessels (**Figure 29**). Therefore, combining the target prediction analysis and validation by qPCR of deregulated miRNAs and mRNAs, miR-144-3p and its predicted target *Dnmt3a* were selected as an age-deregulated pair of interest.

3.3.4 Confirming the age-induced deregulation of *Dnmt3a* protein levels in the female BBB by immunohistochemistry

To determine whether changes in *Dnmt3a* gene expression were also related to protein levels, *Dnmt3a* protein expression was assessed by immunofluorescence of microvessels in brain sections from young and aged female C57BL/6J mice (**Figure 31**). Quantification of *Dnmt3a* staining showed a significant decrease in both staining intensity and percentage area of *Dnmt3a*-positive microvessels of aged mice compared to those of young mice.

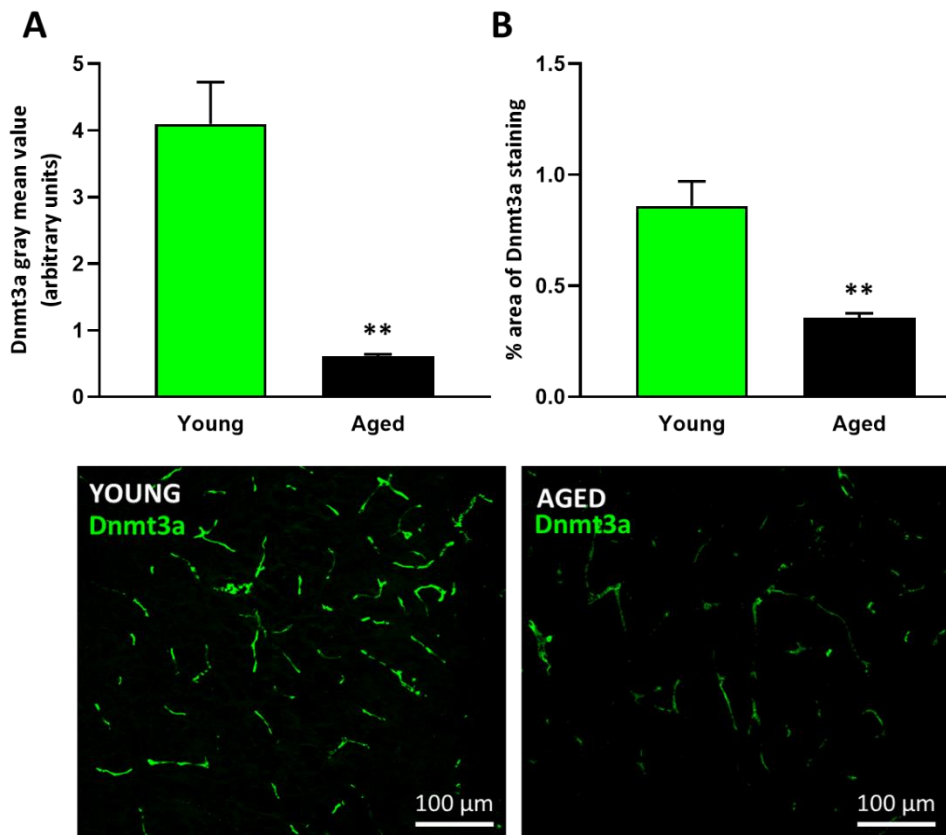


Figure 31. Immunofluorescent staining of *Dnmt3a* in young and aged brain microvessels of female C57BL/6J mice.

Brain microvessels positive for Dnmt3a are shown in green. Quantification of Dnmt3a staining shows a significant decrease in brain microvessels of aged mice in both intensity (A) and percentage of area stained (B). Therefore, these results suggest a decreased Dnmt3a expression at the protein level in aged brain microvessels when compared to young brain microvessels of female C57BL/6J. (n=4/age group, Student's t test; **, $p < 0.01$).

3.4 Discussion

In the present study, age-related BBB changes in gene expression and miRNA deregulation were assessed by MACE-Seq and Small RNA analysis using isolated microvessel from young and aged female C57BL/6J mice. According to our results, the ageing female BBB showed a significant upregulation in genes associated with inflammation and immune response, where the majority of downregulated genes were related to metabolic and signalling pathways. Similarly, a few miRNAs were deregulated in ageing, and their predicted targets were associated with pathways involved in vascular development, cell migration or response to stress. Amongst them, miR-144-3p and *Dnmt3a* were selected for further analysis assessing their putative role and the effect of their deregulation on BEC function. MiR144-3p was observed to be upregulated in ageing. By contrast, *Dnmt3a* mRNA levels were shown to decrease in ageing and Dnmt3a protein expression was also observed to be significantly lower in the aged female brain vasculature.

Transcriptomic analysis of age-related changes at the BBB was carried out using microvessel fragments, which generated a higher yield of RNA but from a mix of cells within the neurovascular unit. Classical approaches to isolating cerebrovascular components include tissue homogenization combined with filtration steps to ensure enrichment of a certain type of blood vessel (Yousif et al. 2007). Additional specificity regarding the selected vascular fraction is achieved by density gradient separation (Bowman et al. 1983; Debault et al. 1979). Techniques such as FACS-sorting or antibody-coated microbeads allow for isolation of single cells and thereby limit the contributing RNA to a single source (Miebach et al. 2006). However, when RNA is extracted from a mix of cells as is the case of the present study, some considerations need to be made when interpreting the findings. Indeed, the exact source of RNA is unknown and the transcriptome of individual cells (i.e. BECs, pericytes) cannot be distinguished, in contrast to other studies that used purified cell fractions with higher specificity in the post-analysis (Daneman et al. 2010). Also, our results showed expression levels of *SMA*-

α and *PDGFR- β* to be decreased in the ageing brain, which may suggest differences in cell composition (pericytes and smooth muscle cells, especially) of the microvessel fraction when comparing young and aged mice. Although changes in cell markers do not necessarily correspond to changes in quantity of specific cells, they need to be considered when analysing sequencing data. For instance, differential starting RNA due to different levels of isolated BECs, pericytes, smooth muscle cells or astrocytes between age groups might lead to consider simple compositional differences as true age-related alterations when assessing relative expression levels.

In addition to the isolation method, the sequencing technique will also influence the analysis. Samples with low-abundant transcripts, such as the ones used in this project, may be beyond the scope of traditional RNA-Seq. In that case, alternative methods such as MACE-Seq, a modification of RNA-Seq, may be of use (Torres et al. 2008; Zhernakov et al. 2019b). For the sequencing analysis it is equally important to consider the advantages and limitations. MACE-Seq provides high resolution gene expression analysis of differentially expressed low-abundant transcripts, however it does so by focusing on the 3'-end of the transcript which limits the reads per sample that can be obtained. Furthermore, our results have shown an age-dependent change in BBB cell proportions, which in addition to the heterogeneous mixture, might affect the interpretation of the sequencing data. A major contribution from certain cell types in the aged sample, but not in the young sample, together with the 3'-focused sequencing, might conceal subtle changes in gene expression and complicate the comparison between age groups. In fact, the relative level of pericyte and astrocyte markers appears to change in ageing, thereby some of the alterations in mRNA levels observed in our data may be due to differential enrichment of cells in the aged microvasculature rather than ageing itself. Interestingly, these qPCR results are consistent with the potential ultrastructural changes addressed more in pericytes than in astrocyte in the previous chapter. Although not completely, alternate tools can be used to address some of these limitations and determine whether a gene of interest is enriched in a certain cell type. These tools include gene expression databases such as the molecular atlas developed by Betsholtz group (He et al. 2018; Vanlandewijck et al. 2018) (<http://betsholtzlab.org/VascularSingleCells/database.html>) which used data from single-cell RNA sequencing analysis to define vascular-associated cells in mouse brain and provided a comprehensive profile for cerebrovascular gene expression classified by cell types. Assessing expression patterns by in silico tools (e.g. databases, sequencing datasets) provides better

understanding of the results and helps making decisions for further analyses. However, the caveats derived from sample purity are harder to solve.

More than 300 genes were identified as deregulated between young and aged microvessel samples, with more genes found to be upregulated genes than downregulated in the aged mice. This prominent upregulation of genes is consistent with recent studies in which mouse ageing is characterized for more upregulated genes than downregulated, in contrast to human ageing (Goodall et al. 2019). In our study, many of the upregulated genes (*Fpr2*, *Ccl5*, *Ccr2*, *Tlr13*, *Itga2*, *Oas1b*) are involved in pathways related to inflammatory response, immune system, leukocyte adhesion and cell activation, whereas downregulated genes are mostly related to metabolic and signalling pathways. Therefore, these results suggest that the brain microvasculature becomes pro-inflammatory in aged female mice in comparison to brain microvessels of young female mice. These findings are in line with the hypothesis of inflammageing, which was firstly defined by Franceschi and colleagues (Franceschi et al. 2000). This theory suggests that the antigenic load and stress throughout a lifespan induces a detrimental effect on the inflammatory response that ultimately promotes a pro-inflammatory state due to the altered balance between anti- and pro-inflammatory modulators (e.g. cytokines) (De Martinis et al. 2005). In line with Franceschi's observations, previous studies have shown that a low-grade pro-inflammatory chronic process contributes to the progression of physiological ageing (Vasto et al. 2007). During ageing, the immune system has been shown to undergo gradual functional alterations, including increase of autoimmune responses and higher levels of circulatory pro-inflammatory cytokines (IL-1 β , IL-6 or TNF- α primarily), which gradually lead to a chronic inflammatory state and may contribute to pathological conditions in age-related diseases (Bruunsgaard 2006; Bruunsgaard et al. 2003; Forsey et al. 2003; Sergio 2008).

Age-induced inflammatory processes have also been described in brain tissue, promoting overexpression of genes related to inflammation and oxidative stress, as well as exacerbation of immune responses and microglia activation that may lead to neurodegeneration (Barrientos et al. 2010; C. K. Lee et al. 2000; Stephenson et al. 2018). Furthermore, the age-related chronic inflammation appears to have a vascular effect as well. For instance, endothelial dysfunction has been linked to vascular inflammation and oxidative stress in the human ageing process (Rodríguez-Mañas et al. 2009), whereas endothelial cells themselves have been reported to increase expression of pro-inflammatory cytokines (Donato et al. 2007). Several

studies have described that the chronic increase in peripheral cytokines during normal ageing induces an inflammatory state in BECs (Krabbe, Pedersen, and Bruunsgaard 2004). In fact, secretion of cytokines (e.g. IL-6, IFN- γ) was observed in BECs of aged mice and non-human primates, where it has been suggested to play a role in BBB breakdown and recruitment of immune cells into the brain (Reyes et al. 1999; Wei et al. 2000). Accordingly, more recent studies have reported ageing to exacerbate BEC senescence inducing inflammation in brain arterial vasculature (Fulop et al. 2018), and other studies have associated this age-induced cerebrovascular inflammation with barrier dysfunction, supporting the idea of an increased pro-inflammatory environment at the ageing BBB (Takechi et al. 2013). In addition, recent studies showed how under chronic stress and inflammation, both pericytes and astrocytes become pro-inflammatory and secrete modulators that may enhance cerebrovascular inflammation (Persidsky et al. 2015; Salminen et al. 2011). These results are very much in line with the upregulation of inflammation-related pathways observed in our sequencing analysis, although the functional effect of this pro-inflammatory state on the BBB requires further attention. Both *in vivo* and *in vitro* studies have reported cytokines such as TNF- α and IL-1 β to modulate expression of TJ proteins (ZO-1, occludin-1, claudin-5) via NF- κ B signal transduction (important transcription factor for pro-inflammatory proteins) or regulation of matrix metalloproteinases (e.g. MMP9, MMP2), alterations that lead to higher BBB leakage and disruption (Trickler et al. 2005; Wu et al. 2010). According to these observations, the changes in pro-inflammatory modulators happening at the ageing BBB would ultimately increase its permeability. In addition, our results also suggest an increase in pathways related to leukocyte adhesion, a phenomenon often described in the context of vascular inflammation (Dos Santos et al. 2005). However, this potential age-related increase of leukocyte adhesion and recruitment is not consistent with other studies reporting increased expression of adhesion molecules (e.g. intercellular cell adhesion molecule 1 (ICAM-1)) in the ageing brain vasculature, but in astrocytes (Miguel-Hidalgo et al. 2007; Xu et al. 2010), or those considering the ageing BBB to be characterized by inflammation but not leukocyte recruitment (Elahy et al. 2015). Nevertheless, potential effects of the pro-inflammatory state described in our sequencing data on BBB function are further assessed in the next chapter.

Although most studies support a general effect of inflammageing at the BBB, sex differences might happen in neuroinflammatory and brain vascular alterations partly due to the role of sexual hormones. For instance, in addition to its reproductive and neuroprotective roles, oestrogen has been found to participate in cerebrovascular inflammation modulation, mainly

in protective roles such as increasing eNOS expression/activation, reducing adhesion molecule expression and leukocyte recruitment across the BBB and decreasing the DNA binding activity of NF- κ B in BECs, therefore hindering pro-inflammatory processes such as cytokine production (Galea et al. 2002; Gavin et al. 2009; Mori et al. 2004). Recent studies have also shown that oestrogen prevents inflammation-derived alterations at the BBB, including downregulation of adhesion molecule ICAM-1 and upregulation of BBB integrity promoter ANXA1, although this effect gets compromised during ageing when reproductive senescence-associated decline of oestrogen happens (Maggioli et al. 2016). Accordingly, previous studies have reported the loss of protective and anti-inflammatory effect of oestrogen on the ageing cerebrovasculature (Maggioli et al. 2016; Sunday et al. 2007), which has also been suggested to correlate with the higher incidence of neurodegenerative diseases and stroke in post-menopausal women (Jamshed et al. 2014; Koellhoffer and McCullough 2013).

Apart from oestrogen, other steroid sex hormones such as progesterone and androgens may play a role in age-related inflammaging contribution to BBB alteration. In fact, progesterone has been reported to participate in neuroinflammation mainly playing a protective role after brain injury and promoting regeneration in young adult rats (Jiang et al. 2009), as well as aged rats (Jiang et al. 2016). Early studies showed how progesterone induces downregulation of cytokines TNF- α and IL-1 β right after brain injury, reducing cerebral oedema (He et al. 2004). Progesterone-induced protection against oedema appears to be mediated by blocking *in vivo* interactions between polymorphonuclear neutrophils and BECs in the BBB (Pascual et al. 2013). Additionally, progesterone protective effects include reduction of MMP-9 and -2 activity and TJ protein degradation which attenuates inflammatory response and BBB disruption after stroke in rats (Ishrat et al. 2010). In contrast to both oestrogen and progesterone, androgens such as testosterone have been reported to increase inflammatory responses in brain vasculature even in the absence of cytokines (Gonzales, Duckles, and Krause 2009). Testosterone is more abundant in males; however, females are sensitive to the hormone. Testosterone in females has been shown to be decreased in reproductive years, followed by a small tendency to increase in ageing, however, no sharp changes are shown in testosterone levels as seen in oestrogen or progesterone during menopause (Davison et al. 2005). The potential role of testosterone in inflammatory responses at the ageing female BBB remains unknown, although it is important to highlight the balance between testosterone and oestrogen in cerebral vessels. Testosterone can be metabolized into estradiol, and the cerebrovascular balance of androgens/oestrogens appears to have an effect

on endothelial function, oxidative stress and cerebrovascular inflammation (Krause et al. 2011). Nevertheless, more information is needed to understand the potential consequence of balance or imbalance of androgens/oestrogens on cerebrovascular function and age-related BBB dysfunction.

According to the findings described above, hormonal decline during menopause would leave the brain and cerebrovasculature vulnerable to the toxic influence of a pro-inflammatory environment enhanced with ageing. Therefore, hormone replacement therapies in post-menopausal women, mainly focused on oestrogen and progesterone, were thought to provide the most beneficial effect and bring back the brain vasculature to a pre-menopausal state, however, there is great inconsistency depending on the hormone used. In fact, randomized trials treating post-menopausal women with oestrogen showed a higher risk of developing ischemic stroke (Wassertheil-Smoller et al. 2003). Similarly, oestrogen therapy has been proved to worsen neural impairment induced by chronic inflammation in aged female rats (Marriott et al. 2002). Also, other studies have shown how oestrogen replacement differently affects the female BBB depending on age, reducing barrier leakage in young mice but increasing it in reproductive senescent mice (Bake and Sohrabji 2004; Chi et al. 2006). Altogether, these observations suggest that oestrogen has a beneficial effect on brain vasculature and BBB integrity in young rodents and women, but it becomes detrimental when they reach reproductive senescence. Although the mechanisms remain unknown, several potential explanations have been addressed. These age-dependent shifts might rely on the time of treatment, since alternate studies have shown how initiating replacement therapy in the period closer to menopausal transition might provide more of a beneficial effect than treatments that start long passed the menopause (F. Liu et al. 2012; Selvamani and Sohrabji 2010). However, it could also depend on the receptors, since early studies showed that oestrogen receptor alpha (ER- α), and not beta (ER- β), is critical for oestrogen-mediated brain protection (Dubal et al. 2001). Also, previous studies have highlighted that expression levels of ER- α may modulate the beneficial or detrimental effects of exogenous oestrogen (Bake, Ma, and Sohrabji 2008), and age has been described to deregulate expression of ER- α and ER- β (Wilson et al. 2002), although their deregulation in brain vessels is not clear. In addition, an enhanced inflammatory state in the aged female brain might be contributing to these differences, especially when referred to higher incidence of stroke in post-menopausal women (Ritzel, Capozzi, and McCullough 2013). Furthermore, recent studies have suggested synergistic effects between age and oestrogen in reproductively senescent females via inflammatory and oxidative stress

processes, such as increased ROS production or Cyclooxygenase-2 activity, that might exacerbate cerebrovascular dysfunction also following brain damage (Deer and Stallone 2016). Interestingly, in the case of progesterone treatments, not only the natural hormone is used but several synthetic progesterone-like steroids as well (e.g. progestins), which may have diverse biological effects (Pluchino et al. 2006). Indeed, combination of synthetic progestins with oestrogen has been reported to show non-beneficial effects (Campagnoli et al. 2005; Wassertheil-Smoller et al. 2003), whereas natural progesterone has been shown to maintain its protective and anti-inflammatory effects when administered after ischemic stroke in aged male and female rodents but not in ovariectomised females (Gibson et al. 2011; Yousuf et al. 2014). This body of research keeps expanding and further work must be done to assess the cellular and molecular mechanisms behind synergistic effects of age and hormone levels on cerebrovascular dysfunction and inflammation, which will also aid in our understanding of pathophysiological states such as stroke.

Transcription profiles can be modulated by epigenetic variations that participate in the regulation of gene expression and include histone and post-translational modifications, RNA-based mechanisms (e.g. miRNA-mediated regulation) or DNA methylation (Matouk and Marsden 2008). In the current study, a significant decrease was observed in both mRNA and protein levels of Dnmt3a. Despite the single fluorescent staining used in the present study showing a significant decrease in Dnmt3a, multiple staining including the vascular marker PECAM-1 or nuclear staining agent DAPI, would have helped showing that Dnmt3a is actually expressed in the vessels, as well as confirming equivalent amounts of cellular material from both age groups. Dnmt3a is a member of the Dnmt family that includes mainly Dnmt1, Dnmt3a and Dnmt3b, whose function is to mediate DNA methylation. Different patterns of DNA methylation have been proved to be essential for mammalian development, acting as a regulatory element that can increase or decrease transcription of certain genes depending on the cell type, the tissue or the stage of the individual (Deaton and Bird 2011; Jones and Takai 2001). Ageing has been linked to aberrant DNA methylation patterns, mostly observed as global genomic DNA hypomethylation and specific hypermethylation of CpG islands in several tissues including the brain (Bollati et al. 2009; Christensen et al. 2009). Moreover, altered DNA methylation normally correlates with deregulated gene expression, either increased or decreased (Mangold et al. 2017); however, there is still little information on how age-related changes in DNA methylation may affect transcriptome or functions of certain tissues such as cerebrovasculature. Accordingly, BBB function may also be modulated by

DNA methylation patterns. Indeed, Sirtuin1 (Sirt1), a factor associated with endothelial cell dysfunction in ageing, has been suggested to regulate expression of several TJ proteins in mice by modulating methylation of their promoters (Stamatovic et al. 2019), as it does in lung (Hasegawa et al. 2013); thus, age-induced decrease of Sirt1 in BECs might have relevant effects on BBB permeability. Similarly, DNA methylation alterations have also been described in cultured murine BECs exposed to A β , promoting overexpression of genes related to amyloid pathology and promoting a vicious cycle of damage that accumulates during ageing and leads to neurodegeneration (Chen et al. 2009). Dnmt expression including *Dnmt3a* appears to be significantly decreased in brain during ageing (Cui and Xu 2018). For instance, the age-dependent decrease in *Dnmt3a* expression observed in our results is consistent with previous studies that showed lower levels of this methyltransferase in neurons of the ageing brain (Siegmund et al. 2007), which may contribute to the age-associated genomic hypomethylation of non-CpG regions (H. Liu et al. 2016).

Transcriptional modulation mediated by miRNAs is normally based on suppression and leads to downregulation of their target mRNAs (Bartel 2004). Interestingly, miRNAs have also been reported to play essential roles in the ageing brain and BBB (Mohammed et al. 2017). For instance, recent studies have reported age-induced overexpression of miR-155-5p, well known miRNA related to neuroinflammation and BBB dysfunction that targets TJ proteins (Lopez-Ramirez et al. 2014), although it did not change in our data. Similarly, miR-107 is downregulated in elderly individuals during early AD stages and has been related to BBB integrity protection by targeting endophilin-1 (W. Liu et al. 2016); however, it was not age-deregulated in our data either. In fact, in the current study, 9 miRNAs were identified from the MACE-Seq analysis to be significantly altered in microvessel fractions of aged versus young animals. Among these miRNAs, miR-144-3p, which was confirmed to be increased in aged microvessels, was identified as a predicted negative modulator of *Dnmt3a* expression. This prediction is consistent with recent reports that also predicted *DNMT3A* to be a target of miR-144-3p in serum samples of women highly exposed to perfluoroalkyl substances (Xu et al. 2020). Our results are also in line with previous studies that have reported a significant overexpression of miR-144 in cortex and cerebellum of ageing non-human primates and AD patients (Persengiev et al. 2011). Additionally, miR-144 has also been related to neuroinflammation in the context of intracerebral haemorrhage in mice, promoting microglia autophagy and inflammation response *in vivo*, which enhances brain damage after injury (Yu et al. 2017). Recent studies have also described miR-144 overexpression after brain injury in

rats, suppressing proteins related to neuroprotective, antioxidant and anti-inflammatory roles such as Nrf2, suggesting a potential role regulating cerebral ischaemia (Chu et al. 2019; Liu et al. 2014). Despite the lack of evidence of miR-144-3p expression in the BBB, previous studies have reported its role in TJ protein regulation in blood-tumour barrier (human glioma), promoting increase in permeability (Cai et al. 2015); thus, something similar could be happening in the brain vasculature.

All of these findings suggest that miR-144-3p is an interesting pro-inflammatory factor with a potential role in BBB dysfunction during ageing. Similarly, Dnmt3a might modulate several processes in the ageing brain vasculature and BBB contributing to transcriptional regulation. These are the main reasons why miR-144-3p and Dnmt3a were chosen, and the effects of their deregulation in BEC function are assessed in the next chapter.

Chapter 4. Role of microRNA-144-3p and DNMT3A in inflammation-mediated alterations to permeability and leukocyte adhesion in the hCMEC/D3 cell model of the human BBB.

4.1 Introduction

In the previous chapter, results of the MACE-Seq analysis showed that hundreds of transcripts were deregulated in the microvessels of aged female mice compared to young animals. Amongst these, miR-144-3p and DNMT3A, which are inversely deregulated in ageing, were suggested to interact according to targeting prediction tools (TargetScan7.2 and miRWalk2.0).

MiRNA-144 (miR-144), which includes miR-144-3p and -5p, is a well-known onco-mediator which has been shown to be deregulated in several types of tumour such as glioma or lung cancer, playing beneficial roles (Cai et al. 2015; Chen et al. 2015). However, miR-144 is also expressed in the CNS and in recent years both its role and deregulation have been strongly linked to neuronal dysfunction in ageing and neurodegenerative diseases (X. Cheng, Ku, and Siow 2013). In fact, miR-144 has been reported to be upregulated in neurons of the ageing cerebellum and cortex of chimpanzees and non-human primates (i.e. rhesus macaques) but also in aged humans with AD (Persengiev et al. 2011). The same study suggested a beneficial role of miR-144 in spinocerebellar ataxia, by suppressing ATXN1, however, high levels of this miRNA might be detrimental in other processes of the ageing brain. Indeed, miR-144-3p suppression has been reported to reduce oxidative neuronal injury by promoting signalling pathways that involve Nrf2 and antioxidant response element (ARE) (Li et al. 2018). For instance, Chu and colleagues showed that miR-144 negatively regulates Nrf2 expression in mouse neurons in the context of oxygen and glucose deprivation and reperfusion-derived injury, which leads to hindered Nrf2/ARE activation and reduced anti-oxidant and anti-inflammatory activities (Chu et al. 2019). Accordingly, additional studies have also reported miR-144 to be overexpressed in aged brains of male rodents related to brain injury and Alzheimer's (Sun et al. 2017). In addition, Cheng and colleagues have also shown that miR-144 suppresses the expression of ADAM10, which participates in the accumulation and clearance mechanisms of A β in the brain, thereby suggesting a role of miR-144 and its regulation in AD pathogenesis (C. Cheng et al. 2013). Furthermore, miR-144-3p

overexpression has recently been reported to contribute to neuron apoptosis and brain injury in male rat intracerebral haemorrhage (Fan et al. 2019).

At the vascular level, miR-144 overexpression in glioma vascular endothelial cells has been reportedly associated with downregulation of occludin, ZO-1 and claudin-5 and increased permeability at the blood-tumour barrier *in vitro* by negatively regulating HSF2, which participates in modulating the expression of these tight junction proteins (Cai et al. 2015). Higher levels of miR-144 in aged male rats have also been associated with angiogenesis, by suppressing the proangiogenic Nrf2, as well as oxidative stress damage of BECs, via downregulation of antioxidative proteins in aged brain microvessels (Csiszar et al. 2014). In addition, miR-144 has also been reported to increase both microglia autophagic activity and neuroinflammation after intracerebral haemorrhage in mice (Yu et al. 2017). However, the implications of its age-induced deregulation in the brain microvasculature and BBB function have not been elucidated.

DNMT3A is a DNA methyltransferase which along with DNMT3B modulates *de novo* methylation while DNMT1 regulates the maintenance of methylation patterns (Delgado-Morales et al. 2017; De Jager et al. 2014). DNMT3A was originally described as an essential regulator for the establishment and stable inheritance of methylation patterns on genomic DNA during early mouse embryonic development (T. Chen et al. 2003). Additional studies have reported DNMT3A to also be important for both neural and hematopoietic stem cell differentiation, which also makes it a key factor in pathological conditions such as acute myeloid leukaemia (Challen et al. 2012). DNMTs are widely expressed throughout the adult and postnatal developing brain in rodents, although they have differential distribution depending on the region. In the case of DNMT3A, it is highly expressed in several regions of the forebrain and hippocampus (Simmons et al. 2013). Accordingly, recent studies in adult male mice have reported that overexpression of DNMT3A in neurons of the prefrontal cortex attenuates anxiety-like behaviour and stress (Elliott et al. 2016). Also in male mice, DNMT3A appears to be essential for associative learning and normal memory formation in neurons of the forebrain, a role that cannot be substituted by DNMT1, for example (M. J. Morris et al. 2014). In the context of ageing, DNMT3A activity has been shown to be altered in human senescent cells, in which this enzyme may play a role determining certain apoptotic pathways mostly in tumour conditions (e.g. colorectal cancer) (Zhang et al. 2011). DNMT3A expression has been shown to decrease in mouse cerebral cortex and other brain regions from birth to adulthood

and during ageing (Kraus et al. 2016). In addition, DNMT3A decreased expression has been described in both mouse cortex and hippocampus during ageing (Cui and Xu 2018). Similarly, loss of DNMT3A in the brain has been associated with cognitive impairment in male mice (Oliveira et al. 2012). Although DNMT3A has not been described to change in the ageing BBB, Kalani and colleagues reported MMP-induced BBB permeability in a mouse brain endothelial cell line during hyperhomocysteinemia to be regulated by miRNA-induced modulation of DNMT3B (Kalani et al. 2014), and since DNMT3A has indeed been shown to change in the ageing mouse brain, it might as well be related to some of the age-related changes observed in BBB function in a similar way to DNMT3B. Also, despite the lack of available data regarding DNMT3A role in neuroinflammation, recent studies have proved that DNMT3A loss leads to activation of inflammatory responses in mouse mast cells *in vivo* and *in vitro*, suggesting an anti-inflammatory role for this methyltransferase (Leoni et al. 2017).

Interestingly, although most of the abovementioned studies were performed in males (human and rodent), some sex differences have been observed in the expression of DNMTs. Indeed, female rats have been shown to express higher levels of DNMT3A in the amygdala during brain development (Kolodkin and Auger 2011). The same study suggests that DNMT3A expression might be regulated by sex steroid hormones, which is consistent with previous studies that reported oestrogen and progesterone to promote downregulation of DNMT3A in the human endometrium during the secretory phase of the menstrual cycle (Yamagata et al. 2009). In contrast, another study performed in mouse brain, has shown that intrahippocampal injection of estradiol in aged female mice induces neuronal overexpression of DNMTs, including DNMT3A, and mediates memory-enhancing effects (Zhao, Fan, and Frick 2010). Together, these findings highlight the potential role of hormones in the establishment of sex differences in DNA methylation, an effect that might change depending on the tissue.

In Chapter 3, genes associated with pro-inflammatory pathways were found to be significantly upregulated in the brain microvasculature of aged female mice. Neuroinflammation has been widely reported to promote BBB disruption and increased permeability via deregulation of TJ proteins (occludin, claudin-5) (Argaw et al. 2012), which is also related to BEC dysfunction and activation of MMPs that promote degradation of extracellular matrix (Cardoso et al. 2012). Additionally, another one of the highlights of CNS inflammation is leukocyte infiltration into the brain parenchyma which in turn depends, among other mechanisms, on leukocyte adhesion to and migration across BECs. According to several

studies, T cells are the predominant population to undergo brain infiltration in neuroinflammation, although neutrophils, granulocytes and macrophages may be present as well (Jin, Yang, and Li 2010; Mracsko et al. 2014; Yilmaz and Granger 2010). Two of the main cell adhesion molecules (CAMs) that participate in leukocyte transmigration are vascular cell adhesion molecule 1 (VCAM-1) and ICAM-1 (Wong, Prameya, and Dorovini-Zis 1999). VCAM-1 and ICAM-1 have been shown to participate in mechanisms related to neuroinflammation and ischemic injury after stroke in the human brain, where they promote activation of endothelial cells and transendothelial migration of leukocytes (Supanc et al. 2011). Pro-inflammatory cytokines commonly related to neuroinflammation such as TNF- α or IFN- γ have been shown to upregulate the expression of VCAM-1 and ICAM-1 in BECs (Stins, Gilles, and Kim 1997). In spite of both of them participating in processes of leukocyte adhesion to brain endothelium, some studies in a model of human BBB have reported that ICAM-1 and VCAM-1 participate differentially depending on the leukocyte population, with both of them mediating migration of T cells, while only ICAM-1 appears to be essential for the migration of polymorphonuclear leukocytes (Wong, Prameya, and Dorovini-Zis 2007). Additionally, several chemokines such as CCL2, CCL4 or CCL5 (RANTES) participate in mediating leukocyte trafficking across the cytokine-activated brain endothelium and may also have an effect on BBB function in neuroinflammation, an effect observed in human BECs cells *in vitro* and in female EAE model mice *in vivo* (Quandt and Dorovini-Zis 2004; Dos Santos et al. 2005; Subileau et al. 2009). Interestingly, although in a LPS-stimulated macrophage cell line, DNMT3A inactivation has been recently reported to promote CCL5 expression, which once again suggests a potential anti-inflammatory role for this methyltransferase (Sano et al. 2018). Nevertheless, although leukocyte adhesion and transmigration are principally associated with pathological conditions such as MS, some studies have also shown an accumulation of T cells in the brain parenchyma of healthy aged mice (Chan-Ling et al. 2007; Xu et al. 2010). Accordingly, adhesion molecules could be overexpressed in the ageing brain due to the inflammatory environment linked to normal ageing (inflammageing), although only reported in astrocytes (Miguel-Hidalgo et al. 2007). In contrast, previous studies have reported increased inflammatory state but no significant increase in adhesion molecule expression or leukocyte migration across the brain vasculature of healthy aged female mice (Elahy et al. 2015). These observations show the ongoing controversy regarding the extravasation of leukocytes into the ageing brain in the absence of pathology.

In summary, recent reports have suggested potential interactions between miRNAs and DNMTs in mediating alterations in BBB integrity (Kalani et al. 2014). In addition, as described above, miR-144-3p and DNMT3A play potential roles in ageing and inflammation, which could be related with age-mediated BBB dysfunction. The aims of this part of the study were to elucidate the consequences of miR-144-3p/DNMT3A deregulation in cerebrovascular function using a simplified *in vitro* human BBB model, hCMEC/D3 cell line, brain endothelial cells originally isolated from the temporal lobe of an adult woman with epilepsy (Poller et al. 2008). Therefore, paracellular permeability and leukocyte adhesion were assessed under miR-144-3p/DNMT3A deregulation conditions.

4.2 Material and Methods

4.2.1 hCMEC/D3 culture conditions

hCMEC/D3 cells were cultured in EBM-2 MV media (#00190860, Lonza, Slough Wokingham, UK). This media was then supplemented with Microvascular Endothelial Cell Growth Medium-2 SingleQuots Kit (#CC-4147, Lonza, Slough Wokingham, UK), which includes 0.025% (v/v) rhEGF, 0.025% (v/v) VEGF, 0.025% (v/v) IGF, 0.1% rhFGF, 0.1% (v/v) gentamycin, 0.1%(v/v) ascorbic acid, 0.04% (v/v) hydrocortisone and 2.5% (v/v) fetal bovine serum (FBS); hereafter this media is referred to as EBM-2 Full Cell (FC) media. Cells were seeded onto previously collagen-coated flasks of different sizes (Greiner Bio-one, Gloucestershire, UK) and maintained at 37 °C in 95% air and 5% CO₂ until confluence. For the coating, collagen type I (#C3867, Sigma-Aldrich, Dorset, UK) stock solution was diluted 1:20 in Hank's balanced salt solution (HBSS) (#H9269, Sigma-Aldrich, Dorset, UK) and left for 1 hr at room temperature (RT) or 30 min at 37 °C on the plastic surface of the flask. After the incubation time, collagen solution was removed, and the flasks were filled with EBM-2 FC media in preparation for cell culture.

For those experiments only focused on assessing the levels of mRNA and miRNA expression after transfection, cells were plated on 12-well plates (#665180, Greiner Bio-one, Gloucestershire, UK). In the experiments that used transwell polyester membrane inserts (0.4 µm pore, 12mm diameter, #CLS3401-48EA, Corning Costar, Sigma-Aldrich, Dorset, UK), filters were first coated with collagen type I as previously described for flasks and left in 12-

well plates. Afterwards the collagen was removed, and the inserts were coated with fibronectin (#F1141, Sigma-Aldrich, Dorset, UK). Fibronectin stock was diluted 1:100 in HBSS and left for 1 hr at RT on the already collagen-coated filters. As before, once the incubation time was finished, fibronectin was removed, and cells diluted in the EBM-2 FC media were seeded onto the inserts. For those experiments assessing adhesion, Ibidi cell chambers μ -slide VI 0.4 (#80606, Ibidi ® GmbH, Martinstreid, Germany) were used. Each chamber had six channels that were coated with collagen as described for 1 hr at RT. Collagen was removed and cells diluted in EBM-2 FC media were seeded in each channel.

4.2.2 hCMEC/D3 culture maintenance

Human hCMEC/D3 were grown until 80-90% confluence ($\sim 1 \times 10^5$ cells/cm²) and culture media was changed every 2 days. For splitting, hCMEC/D3 cells were washed in HBSS without Ca²⁺ and Mg²⁺ (#55021C, Sigma-Aldrich, Dorset, UK) a couple of times and then incubated with porcine trypsin-EDTA 0.25% (#11570626, ThermoFisher Scientific, Loughborough, UK) at 37 °C for 4-5 min until detached. Fetal bovine serum (FBS) solution (#12103C, Sigma-Aldrich, Dorset, UK) was then added to neutralise trypsin activity. Cells were counted using a hemocytometer and split as 1×10^6 cells per flask. For all experiments, hCMEC/D3 cells between passage (p) 25 and 34 were used.

4.2.3 T cell (Jurkat) culture conditions

Immortalised T lymphocyte cell line Jurkat (Jurkat cells) (Dr V Male, Cambridge University) were grown in RPMI 1640 w/GLUTAMAX I culture media (#R8758, Sigma-Aldrich, Dorset, UK), supplemented with 10% FBS (#12103C, Sigma-Aldrich, Dorset, UK) and 100 μ g/ml + 100 U/ml of Penicillin/Streptomycin (#15140-122, ThermoFisher Scientific, Loughborough, UK); hereafter referred to as RPMI Full Cell (FC) media. Jurkat cells were cultured in suspension in 75 ml flasks (Greiner Bio-one, Gloucestershire, UK) and 95% air, 5% CO₂ at 37 °C.

4.2.4 *T cell (Jurkat) culture maintenance*

Jurkat cells were maintained at a concentration of $0.3\text{-}0.5 \times 10^6$ cells/ml. Volume was readjusted to fit the proper concentration every 2 days and media was changed once per week. To change media, Jurkat cells were spun down (190g, 5 min) and resuspended in a known volume of RPMI FC media. Cells were counted and resuspended in RPMI FC in order to reach 0.3×10^6 cells/ml. Once every 3 weeks, Jurkat cells were renewed.

4.2.5 *MicroRNA (miRNA) and siRNA transfection*

For transfection, hCMEC/D3 cells were seeded at different concentrations depending on the technique. Cells had to reach 70% confluence 24 hours after seeding, therefore the seeding densities varied between experiments and growth surface used (i.e. 12-well plates, inserts or Ibidi chambers).

At the end of seeding day, EBM-2 FC media was replaced with EBM-2 MV media supplemented with same components as EBM-2 FC media except for antibiotics (gentamycin). The following day, with cells at 70% confluence, transfection of either miRNA precursors (pre-MiRs) or antagonists (anti-miRs) and silencing RNAs (siRNAs) was performed. For hsa-premiR transfections, siPORT Polyamine Transfection Agent (#AM4502, ThermoFisher Scientific, Loughborough, UK) was used, whereas for hsa-anti-miR and hsa-siRNA transfections, Lipofectamine 2000 Transfection reagent (#11668019, ThermoFisher Scientific, Loughborough, UK) was used. Cyanine-3 (Cy3)-labelled controls were also used to test transfection efficiency. The protocol used for transfections is shown in **Figure 32**. hCMEC/D3 cells were transfected either with pre-miR144-3p, anti-miR144-3p (#PM11051 and #AM11051, ThermoFisher Scientific, Loughborough, UK; mature miRNA sequence: UACAGUAUAGAUGAUGUACU) or silencing DNMT3A (siDNMT3A) (SMARTpool, mixture of 4 siRNAs, no sequence specified, #M-006672-03-0005, Dharmacon, Colorado, USA).

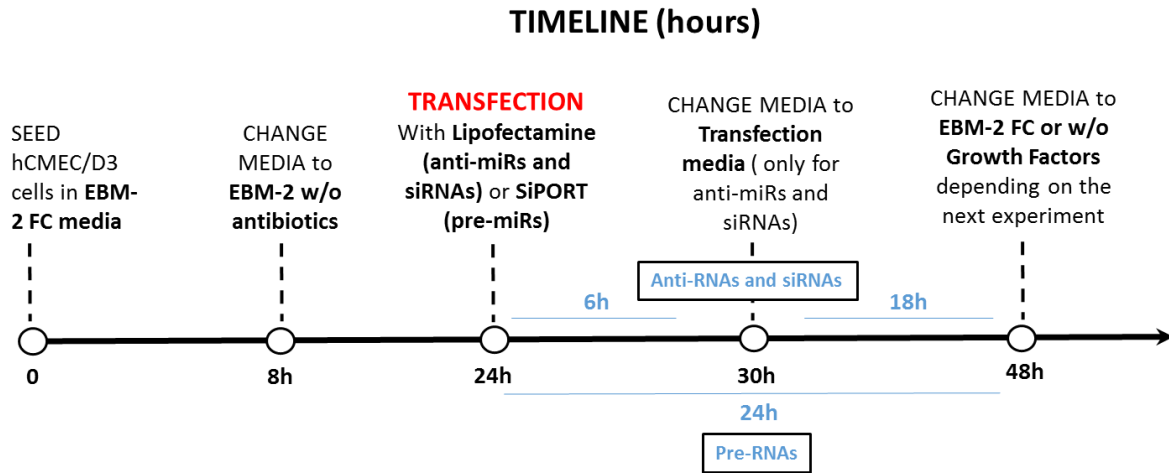


Figure 32. Protocol for transfection of hCMEC/D3 endothelial cells.

On the first day, cells were seeded on a different surface depending on the experiment planned to do. At the end of the day, cell media was changed to EBM-2 media without antibiotics. After 24h, when cells reached 70% confluency, transfection protocol started by incubation with pre-miR-144-3p (diluted in siPORT), anti-miR-144-3p, siDNMT3A (both diluted in lipofectamine) or the scrambled RNAs (diluted in siPORT or lipofectamine). For those cells exposed to lipofectamine, the transfection reagent solution was changed to transfection media only after 6h. Last day, after 24h for the cells in siPORT and 18h for the cells in transfection media only, media was changed to EBM-2 Full Cell (FC) or without growth factors, depending on the following experiment.

In general, transfection agents, siPORT and lipofectamine, were mixed in OptiMEM (#31985070, ThermoFisher Scientific, Loughborough, UK) reduced-serum media (siPORT, 1:25 and lipofectamine, 1:70) to reach the proper concentration according to the supplier's protocol. Cy3-labelled controls, inhibitor or precursor control constructions (scrambled RNA), pre-, anti- and silencing RNAs were also mixed in OptiMEM at the indicated concentration. Afterwards, both the transfection agent and the oligonucleotide were mixed together in EBM-2 MV media supplemented with 0.1% rhFGF, 0.1% ascorbic acid, 0.04% hydrocortisone and 1.25% FBS (hereafter referred to as transfection media) and dispensed onto ~70% confluent hCMEC/D3 cells. Non transfected cells used as negative control were incubated with transfection media only. The final ratio RNA:siPORT in the case of pre-miR-144-3p and precursor control (10 μ M stock) was 1.5 (v/v). In the case of anti-miR144-3p, inhibitor control, siRNA and silencing control (20 μ M stock), the final ration RNA:lipofectamine is 3.3 (v/v).

Regarding oligonucleotides, pre-miR-144-3p, anti-miR-144-3p (1:10 and 1:4) and siDNMT3A (1:4) were used to regulate expression levels of the pair miR-144-3p/DNMT3A. Scrambled RNA (#AM17111 and #AM17010, ThermoFisher Scientific, Loughborough, UK)

and non-targeting siRNAs (#D-001206-14-05, Dharmacon, Colorado, USA), used as negative control of transfection, were undisclosed random sequence RNA oligonucleotides tested in human cell lines and validated to not produce effects on known miRNA and mRNA function by the supplier. Efficiency of transfection was validated using RT-qPCR analysis with the RNA extracted from transfected cells. When using lipofectamine as a transfection agent, due to its higher toxicity, it was necessary to change media to transfection media only 6 hr after starting the protocol. At the end, 24 hr after the start of transfection, media was changed to EBM-2 FC or EBM-2 without growth factors depending on the posterior experiments (adhesion or permeability analysis respectively).

4.2.6 Static paracellular permeability assay in vitro

For static paracellular permeability assay, hCMEC/D3 cells were seeded onto collagen and fibronectin-coated permeable polyester transwell inserts as previously explained in section 4.2.1. Cells were transfected 24h after seeding, when 70% confluence was reached. After 24h, media was changed to EBM-2 media without growth factors and cells were maintained in static conditions in 12-well plates until reaching 100% confluence. The day before, non-transfected cells were either treated with TNF α /IFN γ (1ng/ml, positive control) or left untreated (negative control).

The protocol followed to assess permeability was based on the one used previously in our lab (Lopez-Ramirez et al. 2014). On the same day of experiment, dextran – FITC 70 KDa (#46945, Sigma-Aldrich, Dorset, UK) was diluted in transport buffer made at the lab, 2% (v/v) FBS in DMEM without phenol red, at a concentration of 2mg/ml. A volume of 1.5 ml of the same transport buffer alone was added in 12-well plates, considering 6 wells per condition (insert). Transendothelial electrical resistance (TEER) of hCMEC/D3 cell monolayers was determined in each insert using an Endohm 12 chamber and an Endohmeter (World precision instruments, Florida, USA). Cell media was removed from the upper chamber of each insert and 500 μ l of transport buffer containing the dextran tracer was added. Inserts were then placed in the corresponding first well (out of 6) allocated for each of them and passed to the next one every 5 minutes. After 30 minutes, paracellular flux of the tracer to the lower chamber was assessed by measuring fluorescence in the 12-well plate at λ_{exc} 485 nm and λ_{em} 525 nm using a plate reader (BMG, Ortenberg, Germany). For the posterior analysis, volume cleared was calculated from the fluorescence values of each condition, background (or blank) and the initial

solution (2mg/ml dextran – FITC). Volume cleared values were then plotted against time and the slopes of the curves were used to calculate permeability coefficient of endothelial monolayers (PE, cm/min) as described previously (Tai et al. 2010). When assessing PE in transfected hCMEC/D3 cells, 4 independent experiments were performed, with 3 replicates per condition in each independent experimental repeat (n=4).

4.2.7 Flow-based adhesion assay in vitro

A flow-based adhesion assay was performed using a system previously described by Cerutti et al. (Cerutti et al. 2016). For adhesion, hCMEC/D3 cells were seeded in an Ibidi cell chamber as explained in section 4.2.1 of this chapter. Transfection protocol was followed as discussed previously, 24h after seeding when cells reached 70% confluence. Following transfection, media was changed to EBM-2 FC media and maintained in static conditions. The day before, non-transfected cells were either treated with TNF α /IFN γ (1ng/ml, positive control) or left untreated (negative control).

On the day of experiment, hCMEC/D3 cells were washed with fresh EBM-2 FC media right before and T lymphocyte Jurkat cells were marked with fluorescent green cell tracker CMFDA (5-chloromethylfluorescein diacetate) as indicated by the manufacturer (Invitrogen, Paisley, UK). Cell labelling was performed by incubation with CMFDA during 30 minutes at 37 °C in absence of light. After incubation time, cell concentration was adjusted to 2×10^6 cells/ml.

Thin plastic tubes connected two pumps with the Ibidi chamber. Pre-warmed and degassed EBM-2 FC media was used to establish a flow circuit between the pumps and the chamber. Jurkat cells (leukocytes) were then allowed to flow through the channel with endothelial monolayers and accumulate at 0.5 dyn/cm^2 for 5 min. Afterwards, suspension cell was changed to EBM-2 FC media only and the flow was increased to 1.5 dyn/cm^2 (venular vessel wall shear stress) for 30 seconds to remove leukocytes not firmly adhered on the monolayer. Firm adhesion was defined by leukocytes that adhered on the endothelial monolayer in the field of view (FOV $640 \times 480 \mu\text{m}$) throughout the accumulation time (5 min) and then remained adhered after increasing the flow to 1.5 dyn/cm^2 . Firmly adhered leukocytes were quantified using Image J in 10 different FOVs per channel. Average value was calculated every 10 FOVs. Results were presented as number of Jurkat cells per FOV referred to control.

Images were acquired using 10X objective of an inverted fluorescence microscope (Olympus IX70, Tokyo, Japan) controlled by Image Pro Plus software (Media Cybernetics Inc. Bethesda, USA). This microscope used a Q-IMAGING QICAMFAST 1394 on a 12-bit camera (40 images/min). When assessing leukocyte adhesion onto transfected hCMEC/D3 cell monolayers, 4 independent experiments were performed, with 3 replicates per condition in each independent experiment (n=4).

4.2.8 Flow cytometry

Flow cytometry assay was used to measure efficiency of transfection with Cy3-labelled controls. After transfection, confluent hCMEC/D3 cells were washed in HBSS without Ca^{2+} and Mg^{2+} and harvested using trypsin-EDTA 0.25%. Cells were then washed again, centrifuged at 1500 rpm 5 min and resuspended in a final volume of 500 μl of HBSS. A total of 10,000 events (cells) were counted per experiment using a Becton Dickinson FACScan (Bio-rad, Oxford, UK). Data was later analysed using Cell Quest Software (BD Biosciences, San Jose, CA, USA). To assess transfection efficiency by Cy3-labelling, 3 independent experiments were performed, with 3 repeats or replicates per condition in each independent experiment (n=3).

4.2.9 RNA extraction

Total RNA from isolated cells and microvessel fragments was extracted using miRCURY RNA isolation kit (#300112, Exiqon, Vedbaek, Denmark) as referenced in Chapter 3 of this thesis. The final RNA samples were frozen at $-80\text{ }^{\circ}\text{C}$.

4.2.10 Reverse transcription – Quantitative PCR

RT-qPCR analysis was used to test the successful transfection of endothelial cell as well as to assess expression changes in certain genes (adhesion molecules and inflammation-related factors) following deregulation of miR-144-3p and *DNMT3A*. Genes measured included *DNMT3A* (*Homo sapiens*, F: 5'-ATTACTACGAGGTCAAACCTCC-3'; R: 5'-GGGAAACCAAATACCCTTTC-3'), *CCL5* (*Homo sapiens*, F: 5'-

GTCTTTGTCACCCGAAAG-3'; R: 5'-GACAAGAGCAAGCAGAAAC-3'), and ACTIN- β as reference gene (*Homo sapiens*, F: 5'-GATCAAGATCATTGCTCCTC-3'; R: 5'-TTGTCAAGAAAGGGTGTAAAC-3'). Sequences of ICAM-1 and VCAM-1 primers were not specified. All primers used were pure and simple primers obtained from Sigma-Aldrich (#VC00026, Sigma-Aldrich, Dorset, UK). Same kits and protocols explained in Chapter 3 of this thesis were used. To measure relative expression levels of miR-144-3p, DNMT3A, ICAM-1, VCAM-1 and CCL5 after transfection, 3 independent experiments were performed, with 3 replicates per condition in each independent experiment (n=3).

4.2.11 Relative expression and tags per million normalization

Relative expression by the $\Delta C(t)$ method and normalized expression levels by TPM normalization method were calculated as described in Chapter 3 of this thesis.

4.2.12 Immunofluorescence staining

Immunofluorescence staining was performed as explained in the previous chapter. Frozen brains were sectioned by cryostat at 20 μ m thickness, collected onto slides and stored at -20 °C. Sections were post-fixed by incubation in acetone for 10 min. Sections were then washed with 0.01M PBS (3 times, 5 min) and incubated with citrate buffer (0.01 M Sodium Citrate in 0.01% tween pH 6) at ~90 °C (3 times, 5 min) for antigen retrieval. Unspecific binding sites were blocked by incubation with serum of the antibody host organism diluted in 0.01M PBS for 15 min. Directly after, sections were incubated with anti-VCAM-1 (1:200, #ab134047 rabbit IgG; Abcam, Cambridge, UK) or anti-ICAM-1 (1:200, # MA5407 rat IgG; ThermoFisher Scientific, Loughborough, UK) in 0.01M PBS overnight at 4 °C. The day after, sections were washed with 0.01M PBS (3 times, 5 min) and incubated with AlexaFluor488 anti-rabbit or AlexaFluor555 anti-rat (1:200; Molecular Probes, Eugene, USA) diluted in 0.01M PBS for 2h at RT, protected from light. Slides were coverslipped using Mowiol ® (Sigma-Aldrich, Dorset, UK) containing 0.1% v/v Citifluor (Citifluor Ltd, London, UK) as mounting media. Slides were left in the fridge to dry and sections were later imaged using confocal microscopy Leica confocal N1057 (Leica Microsystems Ltd, Milton Keynes, UK).

To assess immunofluorescence staining of Vcam-1 and Icam-1 in mouse brain tissue, 4 animals per age group were used (n=4/ age group), with 3 experimental repeats each.

4.2.13 Statistical analysis

GraphPad Software (Prism 8.2.0, La Jolla, USA) was used for analysis. Shapiro-Wilk normality test and Q-Q plots were used to assess distribution of the data. Data of transfection efficiency was analysed using one-way ANOVA and Dunnett's correction test. Endothelial permeability coefficient and leukocyte adhesion data was analysed using one-way ANOVA and Tukey's correction test. Finally, relative expression and immunofluorescence data was analysed by unpaired two-tailed Student's t test. Significance is set at $p < 0.05$ and results are presented as mean \pm SEM. The selection of appropriate statistical tests is indicated in each figure legend.

4.3 Results

4.3.1 The role of miR-144-3p in regulating the expression of DNMT3A in human brain endothelial cells (hCMEC/D3)

4.3.1.1 hCMEC/D3 cells are efficiently transfected and transfectant levels maintained for at least 72h

To mimic the age-related changes in miR-144-3p and *DNMT3A* that were observed in the cerebral microvessels of aged mice via MACE-Seq in the *in vitro* model, hCMEC/D3 cells were transfected with pre-miR-144-3p, anti-miR-144-3p and siDNMT3A. General transfection efficiency was first verified using a Cy3-labelled pre-miR negative control (**Figure 33**). Efficiency of the technique was measured by flow cytometry at 24h, 48h and 72h after performing the transfection with Cy3-labelled controls. As determined by fluorescence microscopy and flow cytometry (**Figure 33A**), over 85% of hCMEC/D3 cells were found to

be transfected (**Figure 33B**). The transfectant was detected as early as 24h following transfection and remained stable for at least 72h post-transfection.

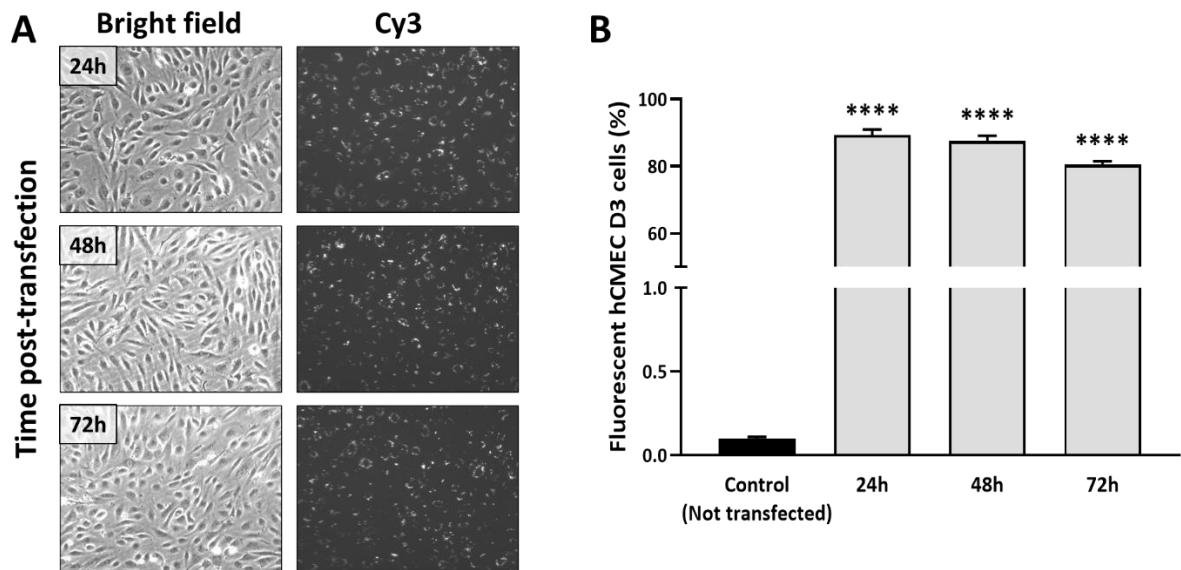


Figure 33. Efficiency of miRNA precursor transfection in hCMEC/D3 cells.

hCMEC/D3 cells were transfected using Cy3-labelled pre-miR negative control. **A**) To determine efficiency of transfection, cells were visualized using bright and fluorescent microscopy and the percentage of fluorescent cells was measured by flow cytometry using FACS. **B**) Over 85% of hCMEC/D3 cells were detected to be transfected for at least 48h. The percentage of positive cells decreased to ~80% at 72h. (n=3, One-Way ANOVA and Dunnett's correction test; ****, $p < 0.0001$).

4.3.1.2 Transfection of hCMEC/D3 cells with miRNA precursors and antagonists successfully modulates expression levels of miR-144-3p

hCMEC/D3 cells were then transfected with either pre-miR-144-3p or anti-miR-144-3p (**Figure 34**). Levels of miR-144-3p were increased by more than 50-fold in cells transfected with pre-miR-144-3p compared to cells transfected with scrambled pre-miR (**Figure 34A**). By contrast, the relative expression of miR-144-3p was decreased by 20-fold in cells transfected with anti-miR-144-3p compared to control cells (**Figure 34B**).

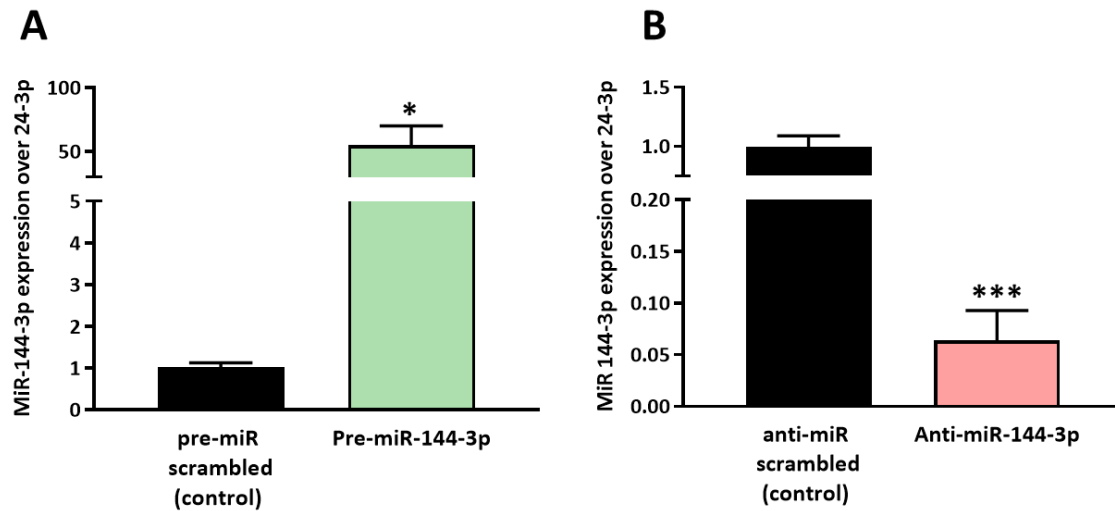


Figure 34. Relative expression of miR-144-3p in hCMEC/D3 cells transfected with pre-miR-144-3p and anti-miR-144-3p.

hCMEC/D3 cells were transfected with pre- and anti-miR-144-3p, whereas scrambled pre- and anti-miR were used as control. Relative expression was calculated over the expression of miR-24-3p as control miRNA. *Actin-β* was used as internal control gene. **A)** Cells transfected with pre-miR-144-3p showed an increase of 50-fold in the relative expression versus those transfected with scrambled pre-miR. **B)** hCMEC/D3 cells transfected with anti-miR-144-3p showed a decrease of 20-fold in the levels of miR-144-3p in comparison to scrambled anti-miR-144-3p transfected cells. (n=3, Student's t test; *, $p < 0.05$; ***, $p < 0.001$).

4.3.1.3 Transfection of hCMEC/D3 cells with silencing RNAs successfully modulates expression of DNMT3A at RNA level

Additionally, hCMEC/D3 cells were transfected using siDNMT3A (**Figure 35**). In those cells transfected with siDNMT3A, the relative expression of *DNMT3A* was significantly decreased compared to cells transfected with scrambled siRNA.

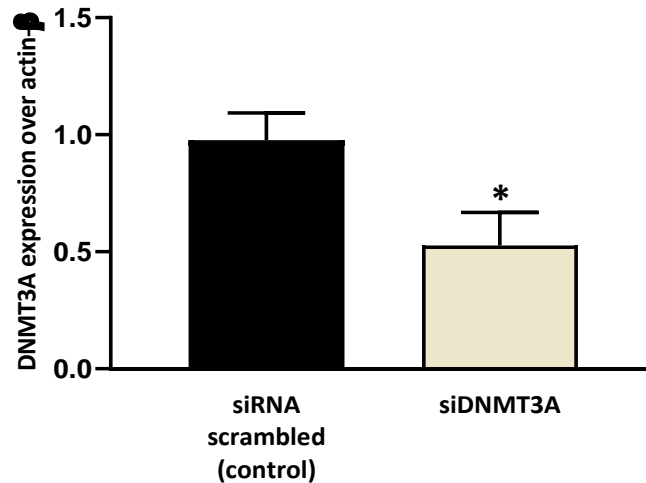


Figure 35. Relative expression of *DNMT3A* in hCMEC/D3 cells transfected with siDNMT3A.

hCMEC/D3 cells were transfected with siDNMT3A, whereas cells transfected with scrambled siRNA were used as control. Relative expression was calculated over the expression of *actin-β* as control gene. Cells transfected with siDNMT3A show a decrease of half in the relative expression of *DNMT3A* when compared to those transfected with scrambled siRNA. (n=3, Student's t test; *, $p < 0.05$).

4.3.1.4 Transfection of hCMEC/D3 cells with pre-miR-144-13p and anti-miR-144-3p modulates changes in the RNA expression of *DNMT3A*

To confirm the predicted targeting relationship between miR144-3p and *DNMT3A*, *DNMT3A* mRNA levels were measured in hCMEC/D3 cells transfected with pre- and anti-miR144-3p (**Figure 36**). Cells transfected with pre-miR-144-3p showed a significant decrease in relative expression of *DNMT3A* compared to control cells (**Figure 36A**). In contrast, *DNMT3A* mRNA expression was significantly upregulated in cells transfected with anti-miR-144-3p compared to control cells transfected with scrambled anti-miR (**Figure 36B**).

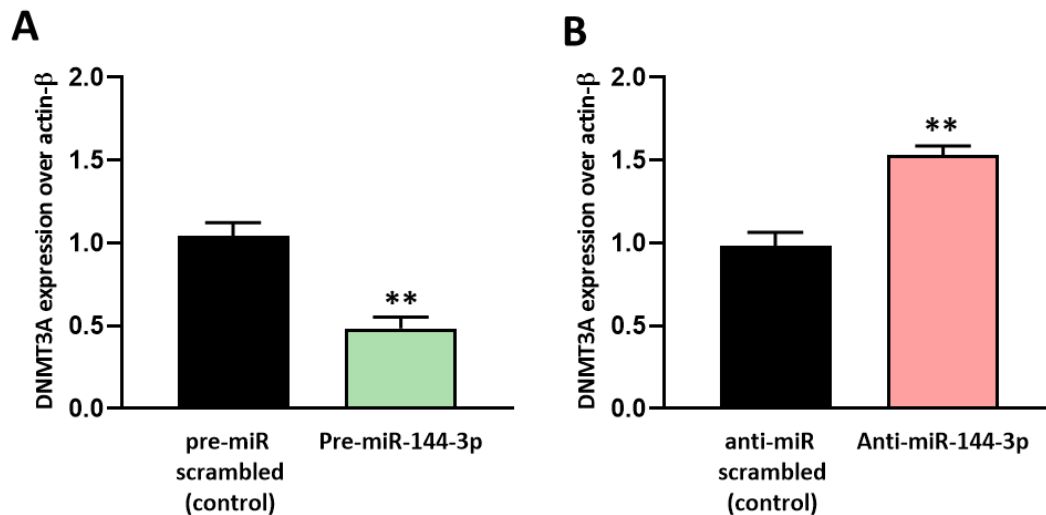


Figure 36. Relative expression of *DNMT3A* in hCMEC/D3 cells transfected with pre-miR-144-3p and anti-miR-144-3p.

DNMT3A mRNA expression levels were measured in hCMEC/D3 cells transfected with pre- and anti-miR144-3p. Relative expression was calculated referred to expression in control cells transfected with scrambled pre- and anti-RNA. Actin-β was used as internal control gene. **A**) In cells transfected with pre-miR-144, relative levels of miR-144-3p showed a significant decrease from 1 to 0.5 compared to control cells. **B**) Cells transfected with anti-miR-144-3p showed a significant increase from 1 to 1.5 in relative expression of *DNMT3A* in comparison to control cells transfected with scrambled anti-miR. (n=3, Student's t test; **, $p < 0.01$).

4.3.2 Paracellular permeability of hCMEC/D3 cells does not change following miR-144-3p deregulation

To determine if changes in miR-144-3p and/or *DNMT3A* expression contributed to dysregulation of BBB permeability, static paracellular permeability was assessed in hCMEC/D3 cells transfected with pre-miR-144-3p. hCMEC/D3 monolayer showed TEER values around $15 \Omega \cdot \text{cm}^2$. No significant change in paracellular permeability was observed in cells transfected with pre-miR-144-3p compared to control cells transfected with scrambled pre-miR. By contrast, non-transfected cells simulated with TNF-α/IFN-γ for 24h as positive control did show an increase in permeability (**Figure 37**). Based on these results, permeability assay was not repeated with hCMEC/D3 cells transfected with anti-mir-144-3p nor siDNMT3A.

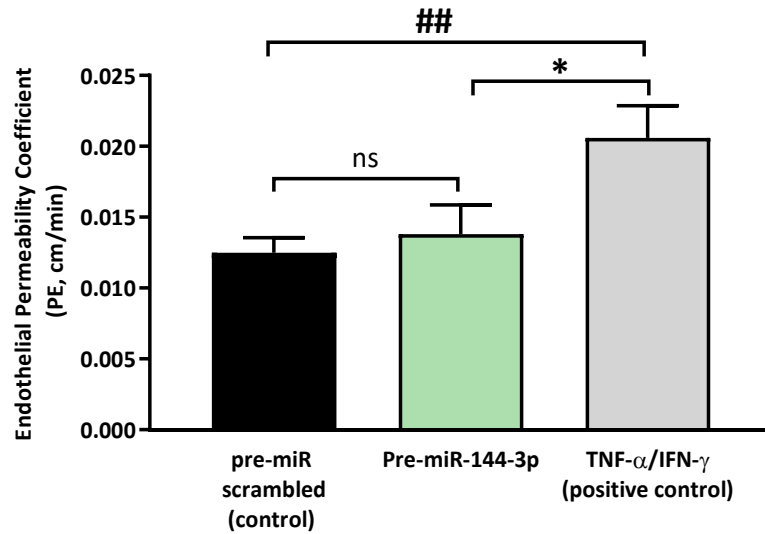


Figure 37. Endothelial Permeability Coefficient (PE) of hCMEC D3 cells transfected with pre-miR-144-3p.

hCMEC/D3 cells are seeded onto transwell inserts and grown until 70% confluence. After 24h, cells were transfected with pre-miR-144-3p and scrambled pre-miR as negative control, and then maintained in culture until 100% confluence (48h post-transfection). Static paracellular permeability was assessed using dextran-FITC 70 KDa and fluorescence was measured in a plate reader. In addition, cells stimulated with TNF- α /IFN- γ for 24h were used as positive control. According to these results, hCMEC/D3 cells transfected with pre-miR-144-3p did not show a significant increase in paracellular permeability compared to cells transfected with scrambled pre-miR. (n=4, One-Way ANOVA and Tukey's correction test; *, $p < 0.05$; ##, $p < 0.01$; ns, not significant).

4.3.3 DNMT3A downregulation promotes a significant increase of leukocyte adhesion to hCMEC/D3 cell monolayer

4.3.3.1 Flow-based adhesion onto hCMEC/D3 monolayer is increased when DNMT3A is downregulated but not when miR-144-3p is upregulated

To determine whether miR-144-3p and/or DNMT3A expression regulates leukocyte adhesion at the BBB, Jurkat T lymphocyte adhesion to hCMEC/D3 cells transfected with pre- and anti-miR-144-3p, siDNMT3A or scrambled pre-/anti-miR and siRNA, was assessed. Non transfected cells stimulated with TNF- α /IFN- γ for 24 hours were used as positive control (**Figure 38**). Leukocyte adhesion was not significantly changed in hCMEC/D3 cells transfected with pre- or anti-miR-144-3p (**Figure 38A and B**). By contrast, a significant increase in T cell

adhesion was observed when *DNMT3A* was silenced by transfecting the cells with siDNMT3A (Figure 38C).

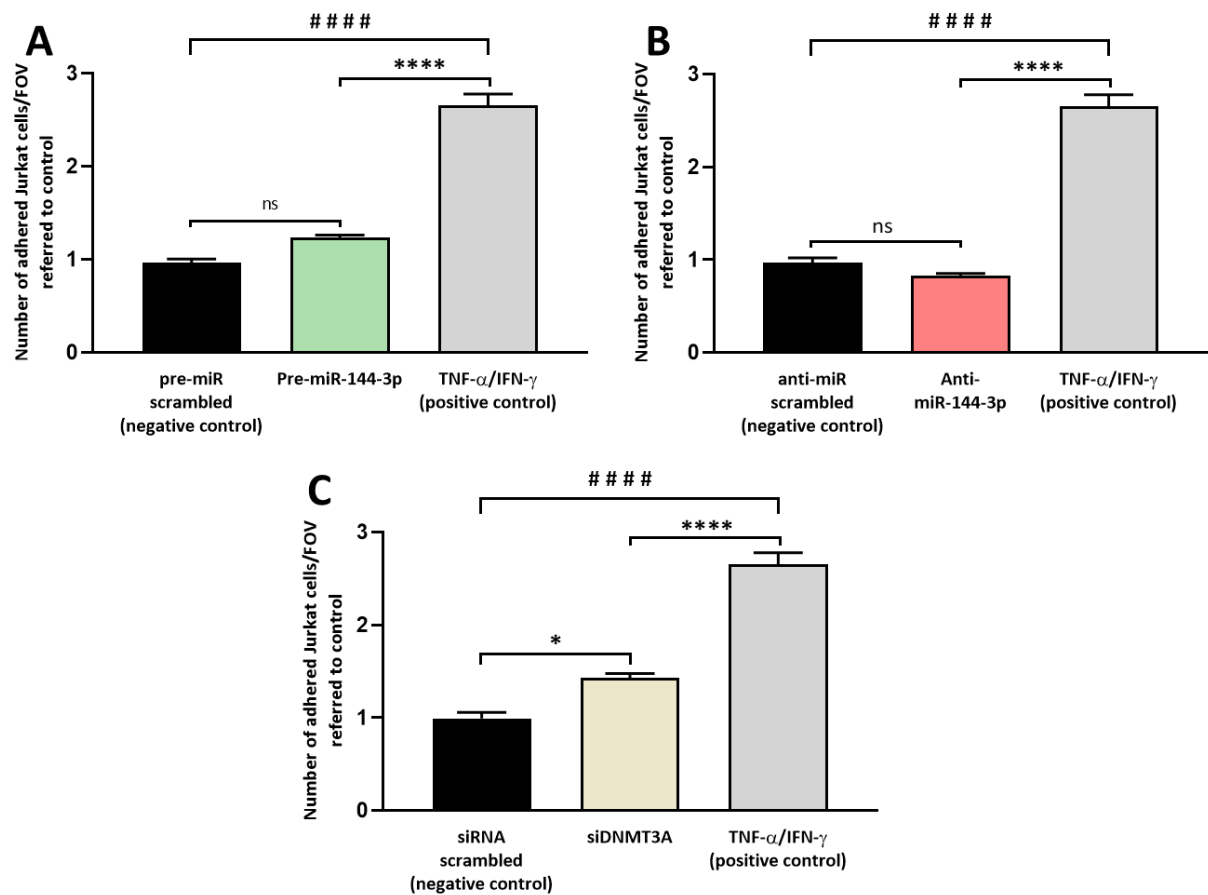


Figure 38. Adhesion of Jurkat T cells to transfected hCMEC/D3 cell monolayer.

hCMEC/D3 cells were transfected with pre-miR-144-3p, anti-miR-144-3p, siDNMT3A and scrambled pre-/anti-miRs or siRNAs as negative controls. Non transfected cells stimulated with TNF- α /IFN- γ were used as positive control. **A**) No significant differences in leukocyte adhesion were observed in hCMEC/D3 cells transfected with pre-miR-144-3p compared to cells transfected with scrambled pre-miR. **B**) Similarly, hCMEC/D3 cells transfected with anti-miR-144-3p did not show significant differences versus cells transfected with scrambled anti-miR-144-3p. **C**) In contrast, hCMEC/D3 transfected with siDNMT3A showed a significant increase in leukocyte adhesion compared to cells transfected with scrambled siRNA. (n=4, One-way ANOVA and Tukey's correction test; ****, #####, $p < 0.0001$; *, $p < 0.05$; ns, not significant).

4.3.3.2 Relative RNA expression level of adhesion molecules VCAM-1 and ICAM-1 is altered with miR-144-3p/DNMT3A deregulation

The mRNA levels of adhesion molecules, *VCAM-1* and *ICAM-1*, were then measured in hCMEC/D3 cells transfected with pre-miR-144-3p, anti-miR-144-3p and siDNMT3A

(**Figure 39**). Relative levels of *VCAM-1*, but not *ICAM-1*, were significantly increased in cells transfected with pre-miR-144-3p compared to cells transfected with scrambled pre-miR (**Figure 39A**). On the other hand, no significant changes were observed in *VCAM-1* and *ICAM-1* mRNA expression in cells transfected with anti-miR-144-3p versus cells transfected with scrambled anti-miR (**Figure 39B**). By contrast, silencing of *DNMT3A* resulted in a significant increase in both *VCAM-1* and *ICAM-1* mRNA levels compared to cells transfected with scrambled siRNA (**Figure 39C**). Together these results support the idea of a functional change in leukocyte adhesion process when miR-144-3p/*DNMT3A* are deregulated that may be mediated by differential expression of *ICAM-1* and *VCAM-1* as part of a complex mechanism.

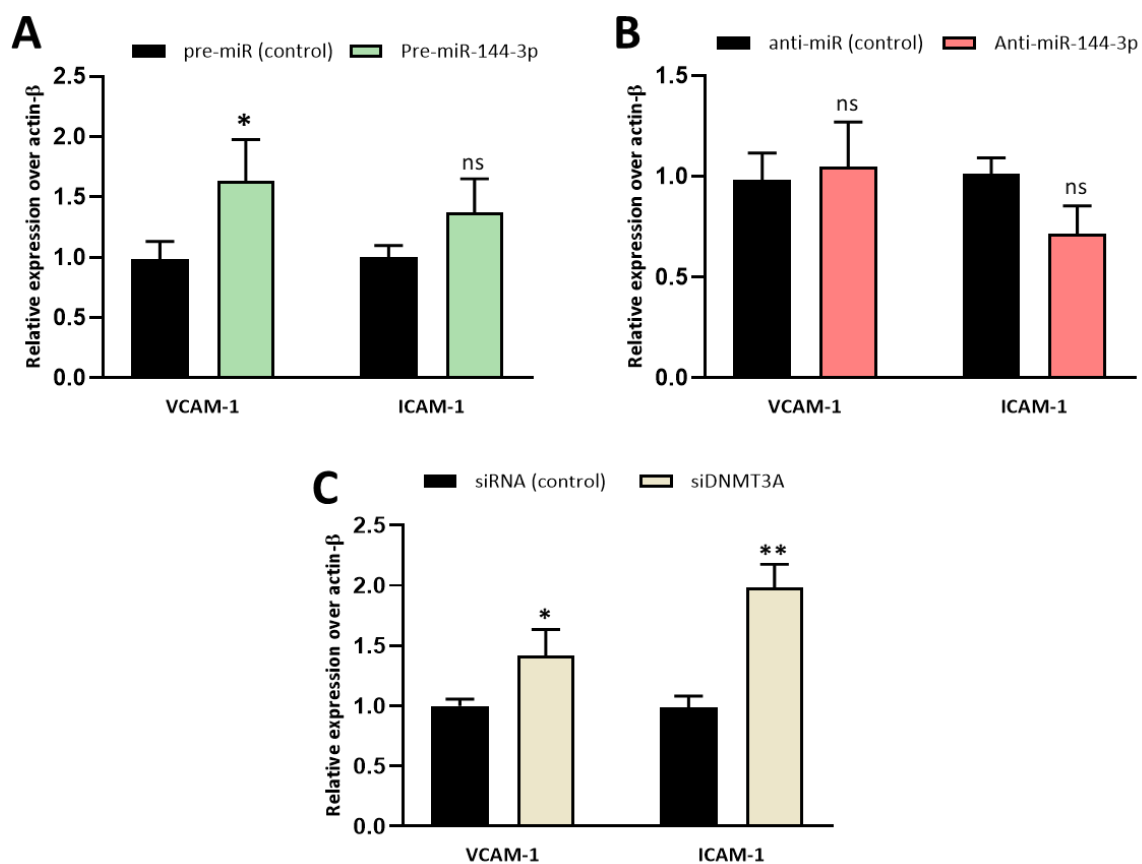


Figure 39. *VCAM-1* and *ICAM-1* mRNA expression levels in transfected hCMEC/D3 cells.

hCMEC/D3 cells were transfected with pre-miR-144-3p, anti-miR-144-3p, siDNMT3A and scrambled pre-/anti-miRs or siRNAs as negative controls. **A**) *VCAM-1*, but not *ICAM-1*, was significantly increased in cells transfected with pre-miR-144-3p. **B**) hCMEC/D3 cells transfected with anti-miR-144-3p did not show any significant differences in *VCAM-1* and *ICAM-1* expression. **C**) The mRNA levels of both *VCAM-1* and *ICAM-1* were significantly increased when *DNMT3A* was directly silenced by siDNMT3A transfection in hCMEC/D3 cells. (n=3, Student's t test; **, $p < 0.01$; * $p < 0.05$; ns, not significant).

4.3.3.3 Relative RNA expression of *CCL5* is increased when DNMT3A is directly silenced in hCMEC/D3 cells but does not change after miR-144-3p deregulation

CCL5 is a chemokine related to inflammation and leukocyte migration and was one of the highest upregulated genes in the MACE-Seq dataset. To determine whether expression of *CCL5* was also induced by miR-144-3p and/or *DNMT3A*, *CCL5* levels were measured in transfected hCMEC/D3 cells (**Figure 40**). No significant differences were observed between cells transfected with pre- or anti-miR-144-3p and their respective scrambled controls (**Figure 40A and B**). However, when *DNMT3A* was downregulated using siDNMT3A, *CCL5* mRNA levels showed a small but significant upregulation (**Figure 40C**).

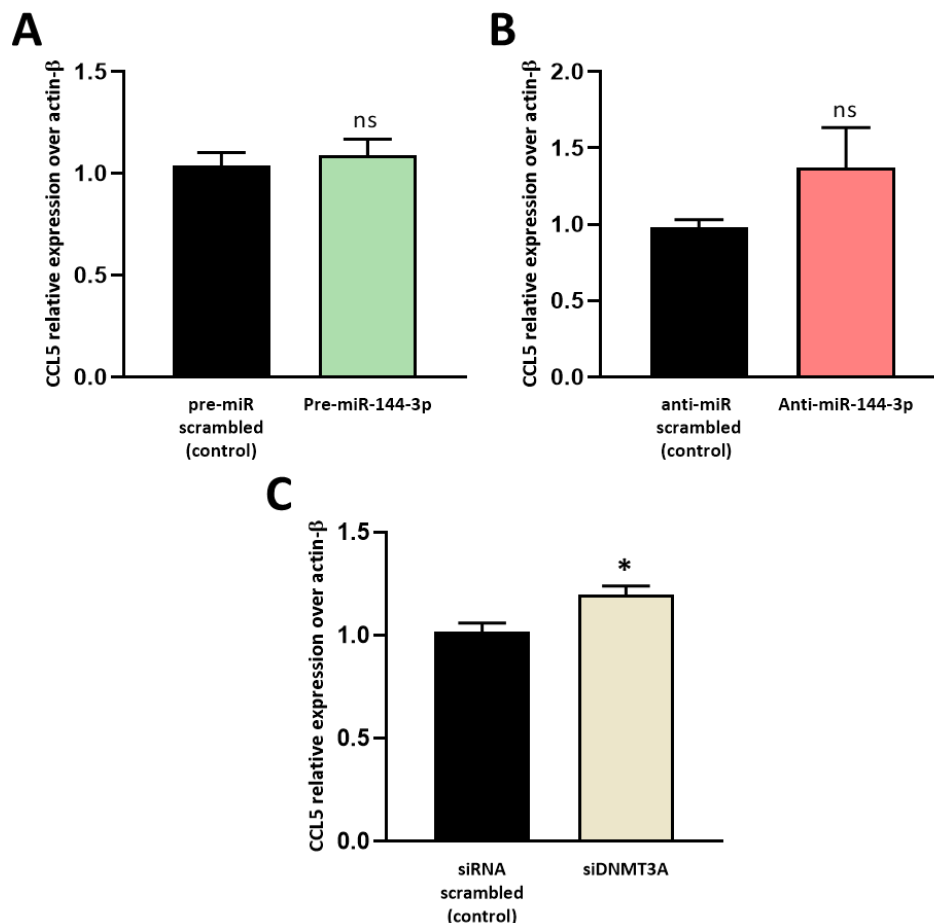


Figure 40. *CCL5* mRNA expression levels in transfected hCMEC/D3 cells.

hCMEC/D3 cells were transfected with pre-miR-144-3p, anti-miR-144-3p, siDNMT3A and scrambled pre-/anti-miRs or siRNAs as negative controls. **A)** hCMEC/D3 cells transfected with pre-miR-144-3p did not show significant changes in *CCL5* expression versus cells transfected with scrambled pre-miR. **B)** *CCL5* mRNA levels were not significantly changed in cells transfected with anti-miR-144-3p compared to cells transfected with scrambled anti-miR. **C)** A small but significant increase in *CCL5* mRNA expression was observed in cells

transfected with siDNMT3A compared to cells transfected with scrambled siRNA. (n=3, Student's t test; *, $p < 0.05$; ns, not significant).

4.3.4 Adhesion molecules in aged female mice microvessel fragments

4.3.4.1 RNA expression levels of *Icam-1* and *Vcam-1* are not changed in microvessel fragments of aged female C57BL/6J mice

To determine if *Icam-1* and *Vcam-1* levels were also altered in the brain microvessels of aged female mice, mRNA levels of both were measured by MACE-Seq (**Figure 41A and B**) and qPCR (**Figure 41C and D**), in order to validate. As shown in **Figure 41**, neither *Icam-1* or *Vcam-1* expression at RNA level were altered between young and aged mice as determined by MACE-Seq and validated by qPCR.

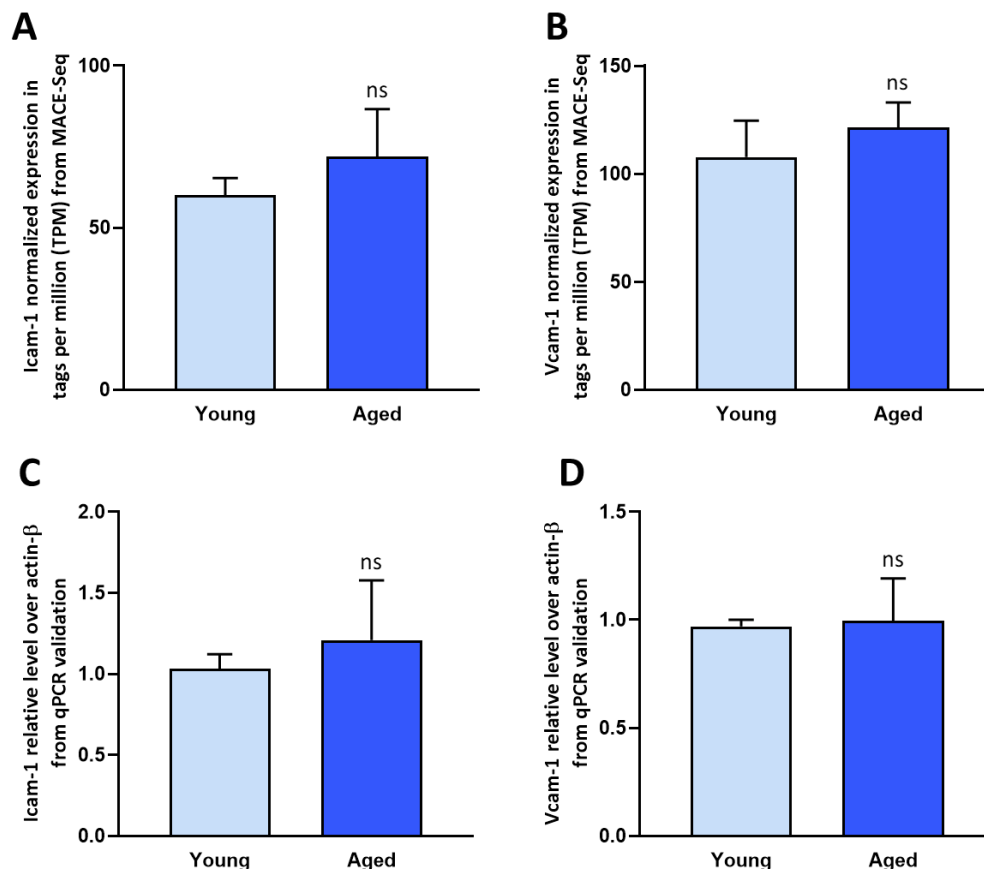


Figure 41. *Vcam-1* and *Icam-1* mRNA expression levels in brain microvessel fragments from female C57BL/6J mice.

Vcam-1 and *Icam-1* RNA levels were measured by both sequencing and qPCR techniques. **A, B)** *Icam-1* and *Vcam-1* expression did not show significant changes in aged brain microvessels compared to young capillaries in MACE-Seq analysis. **C, D)** Similarly, *Icam-1* and *Vcam-1* RNA relative expression is not significantly modified in aged brain microvessel fragments when compared to young brain microvessel fragments. (n=3, Student's t test; ns, not significant).

4.3.4.2 *Icam-1* but not *Vcam-1* protein expression is changed in aged microvessel fragments of female C57BL/6J mice

Levels of protein expression of *Vcam-1* were assessed by immunohistochemistry in cortical brain sections from young and aged female C57BL/6J mice (**Figure 42**). No significant differences were observed in intensity (**Figure 42A**) or % area positive for *Vcam-1* protein expression (**Figure 42B**) between brain microvessels of young and aged female mice.

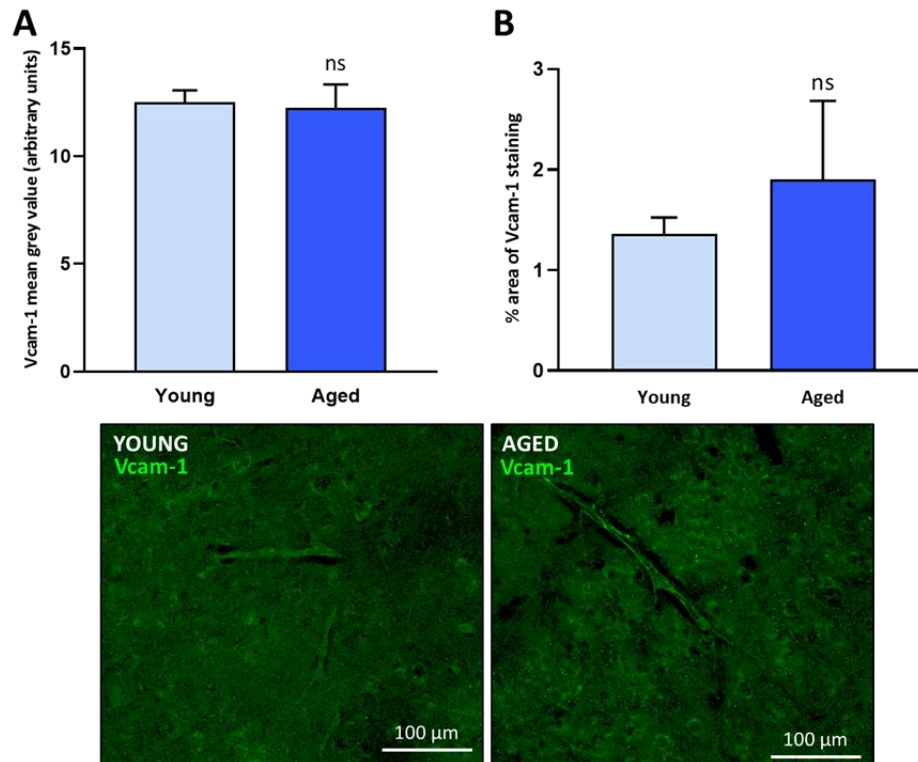


Figure 42. Immunofluorescent staining of *Vcam-1* in young and aged brain microvessels of female C57BL/6J mice.

Brain microvessels positive for *Vcam-1* were identified as green. Measurement of *Vcam-1* staining in young and aged microvessels did not show significant differences in **A)** intensity or **B)** percentage of area stained from young to aged. (n=4/age group, Student's t test; ns, not significant).

Levels of protein expression of Icam-1 were also assessed by immunohistochemistry in cortical brain sections from young and aged female C57BL/6J mice (**Figure 43**). Both staining intensity (**Figure 43A**) and % area positive for Icam-1 protein expression (**Figure 43B**) were significantly increased in aged microvessels compared to young microvessels.

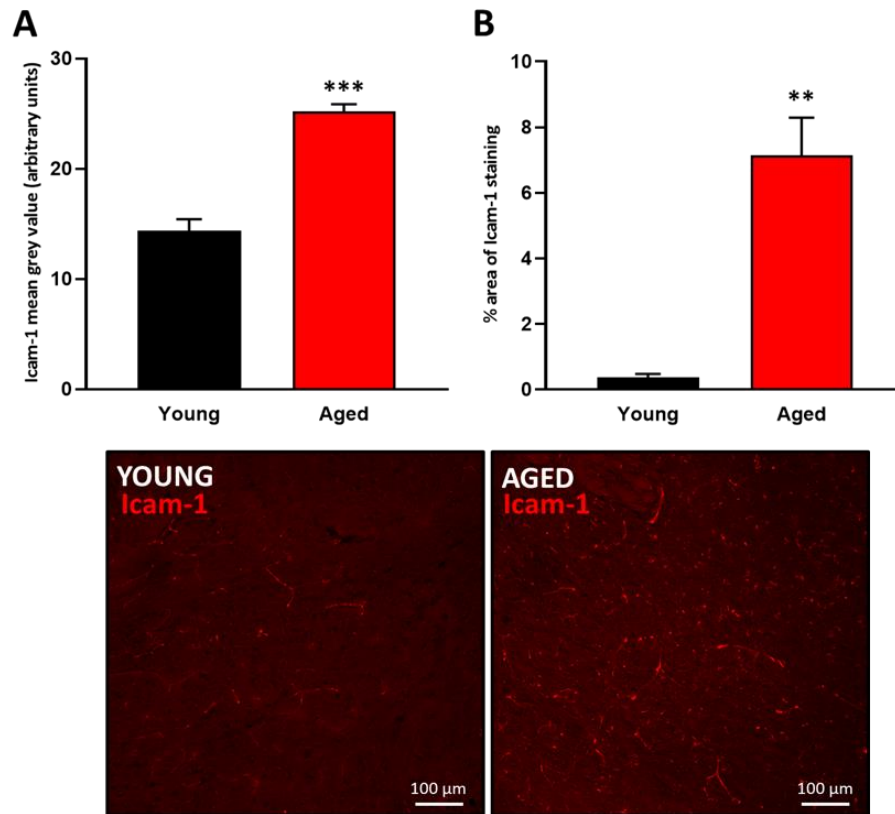


Figure 43. Immunofluorescent staining of Icam-1 in young and aged brain microvessels of female C57BL/6J mice.

Brain microvessels positive for Icam-1 were identified as red. Measurement of Icam-1 showed a significant increase in both **A**) intensity and **B**) percentage of area stained in aged brain microvessels compared to those in young mice. (n=4/age group, Student's t test; **, $p < 0.01$; ***, $p < 0.001$).

4.4 Discussion

In this chapter, we aimed to confirm the interrelation between miR-144-3p and *DNMT3A* in hCMEC/D3 and assess the effect of their deregulation in paracellular permeability and leukocyte adhesion to BEC monolayers. Altogether, our findings support the hypothesis that miR-144-3p regulates *DNMT3A* expression in brain endothelial cells, and that *DNMT3A*

in turn regulates levels of inflammatory mediators. Alterations in miR-144-3p expression did not affect static BBB permeability or leukocyte adhesion. However, decreased expression of *DNMT3A* was associated with an increase in leukocyte adhesion in human BECs. This increase in adhesion was concomitant with increased RNA levels of *VCAM-1*, *ICAM-1* and *CCL5* in hCMEC/D3 cells. Protein expression of Icam-1 but not Vcam-1 was significantly increased in aged female brain microvessels compared to young animals. These results suggest that the age-related increase of miR-144-3p negatively regulate *DNMT3A* expression, and *DNMT3A* independently affects DNA methylation patterns in BECs leading to overexpression of pro-inflammatory factors such as *ICAM-1*, *VCAM-1* and *CCL5*, contributing to an increase in leukocyte adhesion to the endothelial cells of the BBB.

Target prediction analysis of transcriptomic data generated from the MACE-Seq experiments showed *DNMT3A* as a predicted target of miR-144-3p. Consistent with the *ex vivo* analyses in female mouse tissue, levels of *DNMT3A* RNA were found to change in the opposite direction to miR-144-3p in human BECs transfected with pre- and anti-miR-144-3p. These results confirm the reciprocal relationship between miR-144-3p and *DNMT3A* in hCMEC/D3 cells. Age-mediated deregulation of both miR-144-3p and *DNMT3A* in opposite directions have been previously reported in different studies in humans and rodents and mostly occurring in neurons (Cui and Xu 2018; Fan et al. 2019; Persengiev et al. 2011). In addition, although not in the context of endothelial cells or BBB, recent studies in women of different ages exposed to perfluoroalkyl substances have predicted *DNMT3A* as a target of miR-144-3p (Xu et al. 2020). Nevertheless, no correlation between miR-144-3p and *DNMT3A* had been confirmed in the brain cerebrovasculature to our knowledge. Interestingly, miR-144-3p and *DNMT3A* have both been implicated in inflammatory processes: MiR-144-3p overexpression has been reported to correlate with increased neuroinflammation and decreased anti-inflammatory protein expression in male mice and rats (Chu et al. 2019; Yu et al. 2017), whereas *DNMT3A* appears to play an anti-inflammatory role in male and female mice, since its loss or decrease have recently been linked to promote inflammatory activation (Jacquelin et al. 2018; Leoni et al. 2017). Taken together, these observations suggest a role of miR-144-3p and/or *DNMT3A* deregulation in inflammatory processes that may be occurring at the ageing BBB. To determine whether miR-144-3p and/or *DNMT3A* had any effect on BEC function and inflammation, paracellular permeability and leukocyte adhesion were assessed in hCMEC/D3 cells in the context of miR-144-3p/*DNMT3A* deregulation. According to our results, deregulation of miR-144-3p did not change paracellular permeability or leukocyte adhesion to

BECs. In contrast, despite the effect of *DNMT3A* deregulation was not assessed in paracellular permeability, *DNMT3A* silencing was shown to correlate with increased leukocyte adhesion to human BECs. These findings suggest that despite the regulatory relationship established between miR-144-3p and *DNMT3A*, the latter may have a more prominent and independent role in modulating leukocyte adhesion to BECs. In fact, something similar might be happening in paracellular permeability, which we did not assess due to the lack of alteration by miR-144-3p deregulation, however, it could be possible that *DNMT3A*, and not miR-144-3p, has the strongest effect. Nonetheless, we can speculate that miR-144-3p negatively modulates *DNMT3A*, which in turn, independently, contribute to inflammatory processes such as leukocyte adhesion. Consequently, we can speculate that *DNMT3A* plays an anti-inflammatory role in the brain vasculature that would be attenuated due to its age-mediated downregulation. However, additional measurements of paracellular permeability in the context of silencing *DNMT3A* would aid understanding the actual impact of *DNMT3A* deregulation in BBB function. Also, it is important to acknowledge that a TEER of $15 \Omega \cdot \text{cm}^2$ as observed in the present experiment does not represent a restrictive barrier, thereby a different strategy for the BBB model, like including astrocytes or pericytes, or a different method of measuring permeability, might give higher power to the analysis.

In the present study, *DNMT3A* anti-inflammatory role appeared to also have an effect on the expression of several pro-inflammatory mediators related to adhesion. In fact, silencing of *DNMT3A* mRNA in hCMEC/D3 cells was concomitant with overexpression at RNA level of adhesion molecules, *ICAM-1* and *VCAM-1*, and the chemokine *CCL5*. Although no significant effect of miR-144-3p was observed in leukocyte adhesion, cells transfected with pre-miR-144-3p showed a significant overexpression of *VCAM-1*, which might be due to the indirect downregulation of *DNMT3A* mediated by miR-144-3p. *ICAM-1* and *VCAM-1* have been reported to be upregulated in human BECs under inflammatory conditions including exposure to cytokines (Stins et al. 1997). Also, BEC activation mediated by *ICAM-1* and *VCAM-1* has been implicated in neuroinflammatory processes by contributing to leukocyte transendothelial migration after ischemic stroke in the human brain (Supanc et al. 2011), where they allow leukocytes such as T-cells to firmly adhere to the brain endothelium via interaction with integrins (Nagai and Granger 2018). In addition, chemokines such as *CCL5* have been reported to be upregulated under inflammatory conditions or after brain injury in rodents and humans (Arisi et al. 2015; Kiguchi, Kobayashi, and Kishioka 2012). Similarly, *CCL5* was reported to contribute to leukocyte adhesion and migration across the BBB in several species,

including human BECs *in vitro* and female EAE model mice (Quandt and Dorovini-Zis 2004; Dos Santos et al. 2005; Subileau et al. 2009). Additionally, CCL5 has been described to increase in women and men during normal ageing (Mansfield et al. 2012). Interestingly, ICAM-1 and VCAM-1 have been recently reported to be at least partly regulated by DNA methylation, involving NF- κ B signalling, in human atherosclerosis (Hai and Zuo 2016). However, no correlation has been reported between *DNMT3A* and *ICAM-1/VCAM-1*. By contrast, although not in the context of BBB, Dnmt3a downregulation has been previously linked to overexpression of Ccl5 among other chemokines and cytokines in a LPS-stimulated macrophage line in male and female mice (Sano et al. 2018). These results suggest that *DNMT3A* is exerting a direct or indirect suppression on *ICAM-1*, *VCAM-1* and *CCL5* expression and when *DNMT3A* is downregulated, these inflammatory mediators are significantly overexpressed, contributing to leukocyte adhesion to BECs.

Despite the three inflammatory mediators being significantly overexpressed, *ICAM-1* and *VCAM-1* showed a higher fold change than *CCL5*, which made us focus on these adhesion molecules for further measuring their protein expression levels in the female brain. Notwithstanding the significant change in RNA expression of *VCAM-1* and *ICAM-1* in the *in vitro* analysis, results of the MACE-Seq analysis had shown that mRNA of *Icam-1* and *Vcam-1* remained unchanged in both aged and young brain capillaries from female mice. Therefore, to determine potential changes in protein levels of Icam-1 and Vcam-1, immunofluorescence staining was performed in mouse brain cortical sections. The results showed that cerebrovascular Vcam-1 protein expression did not differ between aged and young female mice. In contrast, Icam-1, although not changed at RNA levels, showed a significant increase in protein expression in the ageing female cerebrovasculature compared to young brain vessels. As mentioned for Dnmt3a staining in the previous chapter, a multiple staining including a vascular marker (e.g. PECAM-1) and DAPI, would have helped showing Vcam-1 and Icam-1 location in the vessels, as well as confirming equivalent amounts of cellular material from both age groups. In any case, the findings suggest, first, a differential regulation of Icam-1 and Vcam-1, and second, modifications in Icam-1 at the protein level in the ageing female mouse brain. Differences in Icam-1 and Vcam-1 modulation might be in turn associated with an age-deregulation of Dnmt3a that induces overexpression of Icam-1, but not Vcam-1, in brain microvessels. A differential mechanism of Dnmt3a-mediated regulation might be supported in the present study by a higher overexpression of *ICAM-1* compared to *VCAM-1* when *DNMT3A* was silenced in hCMEC/D3 cells. Interestingly, previous studies have already shown

differences in ICAM-1 and VCAM-1 function, such as differential contribution to leukocyte adhesion and migration across the human BBB *in vitro*, depending on the leukocyte population (Wong et al. 2007). Also, Icam-1 levels on the surface of BECs have been reported to influence the pathway of T cells across the inflamed monolayer, with higher levels of Icam-1 promoting a transcellular pathway (Abadier et al. 2015). Interestingly, these observations might support the theory of different regulatory mechanisms depending on the adhesion molecule. In addition, differences between mRNA and protein levels of Icam-1 during ageing, are pointing at potential post-translational regulation mechanisms. Indeed, several post-translational modifications may alter the structure and function of proteins, going from amino acid changes (e.g. phosphorylation) to the addition of macromolecules (Nalivaeva and Turner 2001). Western blot analysis of Icam-1 protein levels in isolated microvessels of aged and young female mice, in addition to the analysis of potential post-translational modifications in Icam-1 protein structure, might give some insight to its Dnmt3a-mediated regulation. In any case, the age-induced upregulation of Icam-1 observed in our analysis is in contrast with recent studies that described inflammation and TJ alterations in the ageing BBB of female mice, but not overexpression of adhesion molecules or increased leukocyte transendothelial migration (Elahy et al. 2015). However, our results were consistent with studies that have reported an increase in adhesion molecule expression in aged endothelial cells from rodents and humans (Csiszar et al. 2008). Also, previous studies have described a higher expression of Icam-1, but not Vcam-1, in BECs and astrocytes of aged male mice when exposed to inflammatory cytokines (Xu et al. 2010). Nevertheless, further research is needed in this field, and additional experiments measuring leukocyte adhesion in the female cerebrovascular *in vivo* might shed some light on the age-induced crossing of immune cells into the CNS in absence of pathology.

Most of the studies discussed above have reported the same mechanistical differences observed in our study (e.g. age-induced downregulation of *DNMT3A* or upregulation of *ICAM-1/VCAM-1/CCL5* in inflammation) independent on the sex. However, some sex differences might be playing a role in this system, since expression of DNMTs has shown certain differences depending on males or females. Indeed, previous studies have suggested that DNMT3A expression might be regulated by sex steroid hormones (Kolodkin and Auger 2011), including oestrogen/progesterone-mediated downregulation of DNMT3A in women as observed during the menstrual cycle (Yamagata et al. 2009), or upregulation of Dnmt3a in aged female mouse brain after injection of estradiol (Liu et al. 2010). If oestrogen induces upregulation of Dnmt3a in the mouse, we may speculate that changes in oestrogen levels as

observed during reproductive senescence might have an effect on Dnmt3a regulation which in turn, and according to our results, will modulate the expression of adhesion molecules such as Icam-1.

In summary, the present study suggests a potential role of *DNMT3A* in the ageing BBB, both by its targeting relationship with miR-144-3p and its independent regulation of pro-inflammatory mediators such as *ICAM-1*, *VCAM-1* and *CCL5*. However, some extra analyses are needed to determine whether DNMT3A is also related with changes in BBB paracellular permeability. Also, most of the experiments were performed in a very simplified *in vitro* human BBB model; therefore, additional experiments *in vivo* would aid to understand the role of DNMT3A in the complex environment of the BBB and whether its deregulation in ageing affects barrier function in more ways.

Chapter 5. General discussion and conclusions

5.1 Summary of Results

This body of work tested the hypothesis that ultrastructural changes at the female BBB are related to alterations in gene expression of protein-coding mRNAs and miRNAs that regulate BBB integrity and function. Results from the structural analysis (3D reconstruction only), sequencing and functional results are summarised in **Table 3** and **Table 4**, respectively.

Table 3. Summary of ultrastructural changes in the ageing female BBB of cortex and hippocampus (3D reconstruction).

<i>Structural Features</i>	Young vs Aged Female Mice		Cortex vs Hippocampus
	Cortex	Hippocampus	
BM thickness	High in aged capillaries	High in aged capillaries	No difference
BEC mitochondria (Number and volume)	No difference	No difference	No difference
BEC Pseudopods (Number and volume)	High number and volume in aged capillaries	No difference	High number and volume in aged cortical capillaries
Pericyte mitochondria (Number and volume)	High volume in aged capillaries No difference in number	High volume in aged capillaries No difference in number	No difference
Pericyte coverage over BECs	High in aged capillaries	No difference	No difference
Astrocyte coverage over BM	No difference	No difference	No difference
Tight junction tortuosity	High in aged capillaries	No difference	High in aged cortical capillaries
Tight junction complexity	No difference	No difference	Lower in aged cortical capillaries

Table 4. Summary of gene expression alterations in the female cortical BBB during ageing.

Age-deregulated mRNAs (MACE-Sequencing Analysis)			
	Number of genes ($p<0.05$)	Associated pathways ($p<0.05$)	
Upregulated mRNAs	211	<i>Inflammatory response; immune system process; response to stress; leukocyte migration; cell activation; leukocyte cell-cell adhesion</i>	
Downregulated mRNAs	94	<i>Cellular response to hormone stimulus; intracellular signal transduction; circulatory system process; response to stress; homeostatic process</i>	
Deregulated miRNAs (Small RNA Analysis)			
	Number of miRNAs ($p<0.05$)	Associated pathways for predicted targets ($p<0.05$)	
Upregulated miRNAs	4	<i>Intracellular signal transduction; endothelial cell migration; cellular response to stress; programmed cell death</i>	
Downregulated miRNAs	5		
Functional <i>in vitro</i> analysis – Leukocyte adhesion to hCMEC/D3 cell monolayers			
	Leukocyte adhesion (Jurkat cells/field) ($p<0.05$)	Adhesion molecule expression ($p<0.05$)	Chemokine expression ($p<0.05$)
Pre-miR-144-3p transfection	No difference	<i>VCAM-1</i> (higher) <i>ICAM-1</i> (no difference)	<i>CCL5</i> (no difference)
Anti-miR-144-3p transfection	No difference	<i>VCAM-1</i> (no difference) <i>ICAM-1</i> (lower)	<i>CCL5</i> (no difference)
siDNMT3A transfection	Higher	<i>VCAM-1</i> (higher) <i>ICAM-1</i> (higher)	<i>CCL5</i> (higher)
<i>Ex vivo</i> analysis – Expression of adhesion molecules in brain tissue			
	<i>Vcam-1</i> ($p<0.05$)		<i>Icam-1</i> ($p<0.05$)
Young mouse brain tissue	No difference		Low staining intensity and area in brain microvessels
Aged mouse brain tissue	No difference		High staining intensity and area in brain microvessels

In the present study, a series of age-related changes in the female mouse BBB have been identified at ultrastructural and molecular levels. Ultrastructural analyses showed that the

female mouse BBB was modified depending on the age of the individual and the brain region analysed, thus, cortical capillaries appeared to present more changes than hippocampal capillaries in aged animals. Based on these results, sequencing analysis was performed on brain microvessels isolated from cortex only. MACE-Seq analysis showed a significant overexpression of genes participating in pathways related to immune and inflammatory response, cell activation and leukocyte migration. On the other hand, most of the downregulated genes were related to metabolic and signalling pathways. Small RNA analysis showed a total of 9 age-deregulated miRNAs. Amongst them, miR-144-3p and its predicted mRNA target, *Dnmt3a*, were reported to be inversely correlated in ageing, and due to their potential contribution to ageing BBB and neuroinflammation, they were selected for further functional analysis. MiR-144-3p expression was confirmed to participate in the modulation of *DNMT3A* expression in hCMEC/D3 cells, which were also used to estimate the potential effects of miR-144-3p/*DNMT3A* deregulation in cellular function. MiR-144-3p overexpression did not show any significant changes in paracellular permeability. Similarly, no alterations in leukocyte adhesion were observed after miR-144-3p deregulation. By contrast, silencing of *DNMT3A* was shown to increase leukocyte adhesion as well as expression levels of adhesion molecules ICAM-1 and VCAM-1 and the chemokine CCL5. These results do not fully address the original hypothesis directly correlating ultrastructural and molecular changes in ageing. However, they do suggest age-induced ultrastructural changes at the BBB that are more prominent in cortex than hippocampus, in addition to gene expression deregulation that leads to a low-grade increase in neuroinflammation. Although no direct link is established between structural and molecular age-induced changes in this thesis, the amount of data generated is an interesting source of material for future studies to better understand the female mouse BBB in the context of ageing.

5.2 BBB alterations during ageing in the context of reproductive senescence

The specific characteristics of the cerebral vasculature are known to change during ageing. These alterations directly correlate with significant BBB breakdown, cognitive decline or neuroinflammation in several species including human and mouse (Bowman et al. 2018; Fulop et al. 2018; Montagne et al. 2015; Sweeney, Sagare, and Zlokovic 2018). In females, the process of ageing is associated with reproductive senescence, characterized among other events

by a decrease in sex steroid hormone levels (Maffucci and Gore 2006; Mobbs et al. 1984). Oestrogen and progesterone are the main steroid hormones in females, thus have been shown to decrease in ageing. In the present study, oestrogen and progesterone plasma levels were measured to assess potential differences in ageing. A common way to present data from oestrogen and progesterone levels is by the ratio progesterone to estradiol (P/E2) (Bäckström and Mattsson 1975; Baird et al. 1991; Hekimoglu et al. 2010). However, in the case of mice, another method may be determination of acyclicity by cytology and assessment of vaginal epithelium (Byers et al. 2012). In the present study, reproductive senescence was confirmed by cytology. In addition, progesterone and oestrogen levels were measured by ELISA assay in plasma of young and aged female mice. P/E2 ratio was not changed between young and aged mice, which is not consistent with previous reports that have shown an age-related increased in P/E2 ratio (Nelson et al. 1981). The unchanged ratio might be due to the use of ELISA over other more sensitive techniques that have higher specificity regarding samples with low concentration sample (e.g. mass spectrometry) (Nilsson et al. 2015).

Oestrogen and progesterone have been reported to play neuroprotective roles in neuroinflammation, oxidative stress and ischemic injury in human and mouse (Galea et al. 2002; Garay et al. 2007; Gavin et al. 2009), due to the brain being a hormone-responsive organ (Miller and Duckles 2008). In addition, oestrogen has shown neurovascular and BBB protection in physiological conditions by promoting mitochondrial efficiency, reducing oxidative stress, attenuating expression of pro-inflammatory cytokines and adhesion molecules and limiting leukocyte migration across the brain vasculature (Corcoran et al. 2010; Santizo and Pelligrino 1999; Stirone et al. 2005). Similarly, progesterone exerts protection against neuroinflammation after brain injury and promotes regeneration in young and aged female rats (Jiang et al. 2009, 2016). Also, progesterone appears to prevent oedema via downregulation of cytokines after ischemia, and by blocking *in vivo* interactions between immune cells and BECs in the BBB (He et al. 2004; Pascual et al. 2013). In addition, progesterone has also been reported to prevent inflammatory response and BBB disruption by attenuation of MMP activity and TJ degradation in rats (Ishrat et al. 2010). Therefore, age-related changes in hormone levels might influence BBB function in women and female rodents. Whereas progesterone appears to maintain ischaemia-protection functions in aged female rats (Tanaka et al. 2018), oestrogen decline has been reported to promote loss of BBB integrity and altered transport within the brain in female mice (Kastin et al. 2001; Wilson et al. 2008) as well as hinder amyloid- β clearance in the human cortex (Li et al. 2000). Anti-inflammatory effects of oestrogen,

reducing adhesion molecule expression and promoting BBB integrity, also get compromised during ageing (Maggioli et al. 2016; Sunday et al. 2007). Based on these findings, hormone replacement therapies were thought to mainly counteract the loss of oestrogen-derived protection (Zandi et al. 2002). Paradoxically, several studies highlighted the detrimental effects of hormone replacement therapy regarding cardiovascular function, enhanced inflammatory parameters or cancer (Campagnoli et al. 2005; Ridker et al. 1999). Additionally, oestrogen therapy has been shown to increase the risk of developing stroke in post-menopausal women (Wassertheil-Smoller et al. 2003), and to worsen inflammatory-induced neural impairment in aged female rats (Marriott et al. 2002). Oestrogen differentially affects brain vasculature and BBB depending on age, preventing leakage in young rodents but increasing it in aged animals (Bake and Sohrabji 2004; Chi et al. 2006). Potential explanations for this age-dependent role of oestrogen include types of therapies (e.g. transdermal versus oral administration) and the points at which therapy is started (Selvamani and Sohrabji 2010), or age-mediated regulation of oestrogen receptors, ER- α and - β (Wilson et al. 2002). Interestingly, studies in middle-aged female rats have shown how depending on the starting point of the treatment, oestrogen-induced expression of ER- α changes, thereby promoting expression of ER- α preferably in hippocampus right after ovariectomy, whereas it would shift to cortical ER- α when the treatment started several months after ovariectomy (Bohacek and Daniel 2009). Altered receptor expression might be linked to the shifted effect of oestrogen. Progesterone and synthetic progesterone-like steroids (e.g. progestins) are also used in therapy and commonly known as progestogens. Different types of progestogens have been shown to promote different effects, including increased risk of breast cancer or stroke when in combination with oestrogen therapy in post-menopausal women (Campagnoli et al. 2005; Wassertheil-Smoller et al. 2003). By contrast, natural progesterone has been reported to maintain protective and anti-inflammatory effects after ischemic stroke in aged male and female rodents but not in ovariectomised females (Gibson et al. 2011), which suggests that other ovarian factors also contribute to the final response to progesterone treatment. These findings suggest that the type of progestogen used in therapy does have an impact in the risk of vascular damage (Santen et al. 2010). Interestingly, recent clinical guidelines from the International Menopause Society have proposed more positive outcomes regarding hormone therapy. In fact, they have reported that transdermal estradiol, in opposition to oral oestrogen therapy, is not related with higher risk of cardio- or neurovascular complications in elderly women, which suggests that hepatic metabolism of oestrogen during the oral therapy somehow induces its detrimental effects (De Villiers et al. 2013). Also, the use of micronized progesterone, a synthetic progestogen with

identical structure to that of endogenous progesterone, has been shown not to increase the risk of vascular-related pathologies (e.g. thromboembolism or stroke) when alone or in combination with transdermal estradiol in post-menopausal women (Mueck 2012; Simon 2012). In any case, the synergistic effects between ageing and sex steroid hormone roles need further research in order to better understand cellular and molecular mechanisms behind it and how they fit into vasculopathies such as stroke in the female brain.

5.3 Implications of ageing to the BBB ultrastructure

5.3.1 *BM thickness is increased in aged brain capillaries*

Vascular BM has been previously shown to undergo several molecular, morphological and physiological alterations during ageing, mostly focused on protein composition but also lower diffusion and filtration rates as well as loss of vascular elasticity and tone (Candiello et al. 2010; Perlmutter and Helena Chang Chui 1990; Zarow et al. 1997). The ultrastructural analysis performed in this thesis showed a significant thickening of the vascular BM in cortical and hippocampal capillaries of aged female mice. Our results are consistent with other studies that have observed a progressive age-induced increase in cerebrovascular BM thickness of capillaries from both cortex and hippocampus of male and female humans and rodents (Alba et al. 2004; Farkas et al. 2006; Gerrits et al. 2010; Hawkes et al. 2013). Some studies have associated this age-induced thickening of microvascular BM with changes in the proportion of extracellular matrix proteins such as collagen IV or laminins (Ceafalan et al. 2019). Similarly, other studies have reported increasing collagen accumulation in brain microvessels not only during healthy ageing but also in neurodegenerative diseases such as AD or PD (Farkas et al. 2000; Uspenskaia et al. 2004). A more in-depth analysis would be needed to assess which components of the extracellular matrix are changed in the aged brain capillaries. Also, our results were not specific enough to discern between endothelial or astrocytic BM. However, available data suggest that the thickening happens mostly in the astrocytic BM and, therefore, astrocytes may play an important role in such alteration (Wyss-Coray et al. 2000). Also, in our study, astrocytic coverage was increased in the 2D analysis but unchanged in the 3D analysis, suggesting possible complex changes in cellular function that would affect the thickening of the astrocytic BM. Nevertheless, while the causes of BM thickening and the cell types

responsible for it are not fully understood, some of its consequences have already been discussed. Increased BM thickness is linked to higher wall stiffness that leads to reduction in vascular elasticity and contractile force, which ultimately may have an effect on cerebrovascular blood flow (Desjardins et al. 2014). Therefore, progressive BM thickening and reduced blood flow might influence the regulation of exchange mechanisms between blood and brain, and may be associated with decreased nutrient supply and elimination of waste products (Farkas et al. 2006). Accordingly, age-induced increase in BM thickness appears to play a role in impaired perivascular drainage of interstitial fluid, contributing to accumulation of solutes and participating in the onset of actual pathological conditions including cerebral amyloid angiopathy and AD (Hawkes et al. 2011). Altogether, these results suggest that BM thickening is a universal feature in the ageing cerebrovasculature, and it may contribute to and exacerbate several neurodegenerative disorders that have a vascular component.

5.3.2 BECs alterations as a mark of cerebrovascular ageing and inflammation

Alongside microvascular changes, endothelial cells experience molecular and structural modifications during healthy ageing (Brandes et al. 2005). Some studies have reported age-induced dysfunction of endothelial cells, including those of the BBB, which show a predisposition towards increased sensitivity to oxidative stress and higher expression of proinflammatory markers (Enciu et al. 2013; Rodríguez-Mañas et al. 2009). Therefore, some related endothelial characteristics were measured at the ultrastructural level in our analysis, including mitochondrial component. Mitochondria play important roles in pathways related to cellular energy and redox homeostasis, signalling, calcium buffering and programmed cell death, and their structure appear to impact on their function (Kluge, Fetterman, and Vita 2013; Picard et al. 2011). Our ultrastructural analysis included assessment of BEC mitochondrial size and number in cortical and hippocampal capillaries. No significant differences in mitochondrial volume or number per cell were observed in aged BECs compared to young BECs in either cortex or hippocampus in the 3D analysis, although the 2D analysis did show a decrease in both volume and number. Therefore, results from the 3D reconstruction, but not from the 2D analysis, are in contrast to previous observations of decreased size and number as well as decreased fusion and fission activity and/or impaired biogenesis of endothelial mitochondria in aged mice, primates and cultured ageing human endothelial cells (Burns et al. 1979; Hicks et al. 1983; Jendrach et al. 2005). However, other studies performed in male rats have showed no progressing differences in BEC mitochondrial area per section with ageing (Burns et al.

1981). Similarly, studies performed in elderly women and men did not show significant changes in BEC mitochondrial population (P. Stewart et al. 1987). These differentially observed changes add complexity to the understanding of neurovascular mitochondrial regulation in the context of ageing, since they do not appear to be similar between sexes or strains. Interestingly, changes in mitochondrial shape and fusion/fission balance have been linked to mitophagy in ageing (Sun et al. 2015). Indeed, mitochondria might undergo more fission events and face senescence (Scheckhuber et al. 2011) or show more fusion over fission and promote cell survival (Gomes et al. 2011). The differences in our 2D and 3D studies, might point out that potential changes in fusion/fission leads to aberrantly shaped mitochondria, which may appear as decrease in volume and number in 2D images but not when 3D reconstruction is taken into account. The balance that controls mitochondrial shape appears to have a higher impact in age-related modifications on mitochondrial function, although further research is needed to understand the ultimate effect on neurovascular and BBB function and whether sex-differences are at play in that scenario.

BEC luminal surface has been shown to become irregular in some experiments involving accelerated senescent mice, where they show cellular protrusions from the cytoplasm into the lumen (E. Y. Lee et al. 2000). In the present study, similar surface projections were observed to be more prominent in the ageing brain capillaries, therefore, the size and number of these endothelial projections were also assessed in our analysis. Pseudopod were higher in number and volume in aged cortical BECs, when these were compared to either young cortical BECs or aged hippocampal BECs. Interestingly, structures similar to these pseudopods have been reported to appear on endothelial surface under inflammatory conditions in several organs including the brain (Lossinsky et al. 1991; Walski et al. 2002). In fact, similar features have recently been described in cultured human microvessel endothelial cells (e.g. HUVEC or human bone marrow endothelial cells) when stimulated with chemokines (Whittall et al. 2013). Additionally, expression of adhesion molecules or chemokine presentation have been reported to occur in specific projections on the endothelial cell surface that are complementary to the pseudopods observed in our study (Øynebråten et al. 2015). Together, these findings suggest that the observed increase in the number and volume of pseudopods in the ageing female BBB may increase the interaction of BECs with immune cells under pro-inflammatory conditions. Thus, our results might indicate that aged BECs are exposed to a more pro-inflammatory environment in the cortex but not in the hippocampus of female mice. A higher pro-inflammatory state in the female BBB during ageing is supported by previous studies that found

a greater upregulation of genes related to immune activation and inflammation in the aged female brain compared to the aged male brain in mice and humans (Berchtold et al. 2008; C. K. Lee et al. 2000).

Furthermore, both mitochondrial and proinflammatory modifications observed in our study have previously been associated with the role of oestrogen in endothelial function. Indeed, some studies have reported a protective role of oestrogen on mitochondrial efficiency via reduction of ROS and preservation of mitochondrial membrane potential in cerebral microvessels and BECs *in vitro* (Guo et al. 2010). In the case of inflammation, available data have shown how oestrogen promotes vascular function and protection against inflammation by increasing eNOS expression and activation (Gavin et al. 2009). In addition, oestrogen has also been reported to reduce adhesion molecule expression and leukocyte recruitment on the cerebral vasculature and to protect the BBB during inflammation (Maggioli et al. 2016; Mori et al. 2004). Oestrogen signalling is mediated by oestrogen receptors (ER- α and - β), from which ER- α appears to modulate the brain anti-inflammatory activity of the hormone, by inhibiting microglia activation (Vegeto et al. 2003) or inflammatory gene expression in brain macrophages via control of NF- κ B pathway (Ghisletti et al. 2005). ER- α is expressed in BECs and its levels are higher in female rats chronically exposed to oestrogen (endogenous, normal cycling females, or exogenous, oestrogen replacement), which explains oestrogen effects in cerebrovascular function (Stirone, Duckles, and Krause 2003). Additionally, experiments on cultured human BECs have shown that ER- α also mediates oestrogen protective role in endothelial mitochondria (Razmara et al. 2008). Oestrogen receptors have shown differential expression during ageing in specific regions of the rat brain including the cortex (Wilson et al. 2002). Age-related decrease in ER- α might contribute to BBB dysfunction in the elderly, as suggested by recent *in vitro* experiments on ER- α -free HUVEC cell line (Kuruca et al. 2017). Thus, changes not only in oestrogen levels, but also in the expression of its receptors might be contributing to the age-related changes described in BECs in our data. Interestingly, regional differences in this hormone/receptor system could also contribute to the differences observed between cortex and hippocampus in our study. Indeed, the developmental and postnatal rat brain has shown regional patterns in ER- α expression, whose levels are stable in hippocampus throughout time, whereas they tend to change more in brain cortical regions depending on the age (Pérez, Chen, and Mufson 2003). In addition, differential expression of ER- α linked to the shift on oestrogen effects in the BBB integrity of middle-aged female rats (Bohacek and Daniel 2009), shows regional variance, suggesting potential differences between cortex and

hippocampus that may have a higher impact on brain vascular function during ageing and prior to hormone replacement treatments.

Therefore, even though our results regarding oestrogen levels do not show a significant change in aged female microvessels, (which as discussed above, might be due to technical limitations) a similar mechanism might be behind the ultrastructural changes in BEC that we have observed. Taken together, these observations suggest that a combined age-related deregulation in oestrogen concentrations and ER expression might be linked to inflammatory processes at the ageing female cerebrovasculature and may have a higher impact in cortical vessels compared to hippocampal vessels. Also, mitochondrial dysfunction appears to occur at molecular and physiological levels rather than at the ultrastructural level, although further analysis on cell energy metabolism, mitochondrial function and/or oxidative stress regulation would be required to clarify this matter.

5.3.3 Ageing as a modulator of pericyte mitochondria and coverage of brain capillaries

Earlier studies have reported age-induced increase in the size of mitochondrial pericytes in both the cortex and hippocampus of male mice (Hicks et al. 1983), while more recent reports have shown an increase of pericytic mitochondrial size in aged Notch3 mutant transgenic mice, therefore in the context of microvascular pathology such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), the most common form of hereditary stroke disorder (Gu et al. 2012). In any case, such observations are consistent with the age-related increase in the volume of pericytic mitochondria observed in female cortical and hippocampal capillaries in our study. Mitochondrial structure and shape have been described to vary depending on processes of fission and fusion which required precise regulation (Fritz, Weinbach, and Westermann 2003). Fission and fusion regulation has been described as an essential mechanism in the modulation of respiratory function, mitochondrial DNA integrity or cell death (Aerts et al. 2008; Leadsham and Gourlay 2010), and might be altered in ageing (Leduc-Gaudet et al. 2015). Indeed, changes in mitochondrial shape appear to correlate with changes in mitochondrial DNA and/or structure, which is reported to occur during ageing in several tissues (Cienca et al. 2011). Therefore, age-related deregulation of mitochondrial structural dynamics might modulate mitochondrial function in oxidative stress and cell death during progression of normal ageing and neurodegenerative diseases (Ansari and Scheff 2010; Mistry et al. 2013; Morozov et al. 2017). Increased mitochondrial volume

and elongated shape have been previously described in aged oocytes, which also showed decreased energy-producing capability, suggesting a possible connection between both processes (Hao et al. 2009; Simsek-Duran et al. 2013). Moreover, abnormal mitochondrial shapes have also been linked to age-induced alterations in mitophagy (mitochondrial autophagy) mechanisms, which are directly related to cell survival and senescence (Sun et al. 2015). Interestingly, fission/fusion balance may change in the context of mitophagy, and mitochondria may undergo fission events that lead to progressive senescence and cell death (Scheckhuber et al. 2011), or, by contrast, they may show reduced fission and display elongated and network-like morphology, which has the potential to both maintain cell viability and postpone mitochondrial dysfunction in ageing (Gomes et al. 2011). These observations, together with our ultrastructural data, suggest potential age-related modifications in mitochondrial function at the BBB, including pericytes, that translate into structural changes and might have a higher effect on cell viability. Pericytes have been reported to modulate cell communication, TJ formation, BBB integrity and blood flow (Dalkara et al. 2011; Dohgu et al. 2005; Peppiatt et al. 2006), thus, changes in mitochondrial function and cell energy in pericytes may have an effect on BBB integrity via modulation of barrier permeability and disturbances in brain microcirculation. Additional studies on pericyte viability and mitochondrial activity might help to confirm and to better understand whether these structural alterations are indeed related to changes in mitochondrial and cellular function.

In addition, pericyte coverage over brain capillaries was shown to be significantly increased in aged cortical capillaries whereas no changes in coverage were observed in the hippocampus. Our results in female mouse brain capillaries are in contrast with several studies performed in male mice and post-mortem human tissues from men and women that previously reported a decrease in pericyte coverage in the ageing brain, including cortex and hippocampus (Bell et al. 2010; P. Stewart et al. 1987). Age-related loss of pericyte coverage and contact with BECs has been previously linked to higher neurovascular dysfunction (Duncombe et al. 2017). Therefore, the opposite situation as observed in our study would suggest a better outcome regarding BBB integrity and blood flow regulation (Kamouchi et al. 2004; Zechariah et al. 2013). However, the biological meaning of this increased pericytic coverage is unclear, further experiments measuring permeability or microvascular flow *in vivo* would give some understanding of the mechanisms behind it.

5.3.4 TJ tortuosity and complexity as potential markers of BBB integrity in cortical and hippocampal capillaries

TJ proteins have been reported to undergo age-mediated modifications (Elahy et al. 2015; Goodall et al. 2018; Mooradian et al. 2003), having an impact at both functional and structural levels of the BBB. Although we did not assess age-related changes in TJ proteins in our analysis, these had been extensively reported (Bake et al. 2009; Elahy et al. 2015), and we did observe significant age-related differences on TJ structure. Indeed, TJ tortuosity was higher in aged cortical capillaries when compared to young cortical capillaries, whereas no significant differences were observed between young and aged TJs in hippocampal capillaries. In addition, TJ length/complexity did not change with age, but aged cortical capillaries showed more complex TJs, as defined by the length of endothelial cell contact, than aged hippocampal capillaries. However, although significant, the region-dependent change in TJ complexity was very small, thus whether it has any biological meaning is unclear. Little information is known about age-related changes in TJ tortuosity and length/complexity, but shorter and less tortuous TJs have been previously linked to TJ protein alterations in levels and subcellular localisation and to BBB disruption after ischemic stroke in granulin mutant male mice (Jackman et al. 2013). Similarly, although in the context of A β and AD, decreased TJ length has been linked to TJ protein disruption and BBB dysfunction (Park et al. 2017). Liebner and colleagues also showed that changes in TJ morphology may correlate with altered TJ protein expression and distribution in microvessels of human glioblastoma (Liebner, Kniesel, et al. 2000). These observations suggest that less TJ tortuosity leads to higher BBB dysfunction and that functional and molecular changes at TJs may translate into rearrangement of their structure in BECs. Whether the increase in TJ tortuosity that we observed in cortical capillaries compared to hippocampal capillaries is related with better BBB integrity would need further analysis, including assessment of expression patterns of TJ proteins. Interestingly, a series of estradiol treatment experiments in ovariectomised middle-aged female rats have reported age-mediated and region-dependent loss of specific TJ proteins, showing altered paracellular permeability in hippocampus but not in cortex (Bake et al. 2009; Sandoval and Witt 2011). These findings point to TJ regulation mechanisms that depend on the brain region and might be modulated by sex steroid hormones. Thus, a combination of sex- and region-dependent differences could be the explanation behind the structural changes in TJs observed in our study, although additional experiments, such as TJ protein expression analysis, for instance, would lead to more conclusive results.

5.4 Implications of ageing in brain microvasculature gene expression profile

In addition to ultrastructural changes, the BBB is also altered at the molecular and gene expression levels during ageing (Osgood et al. 2017; Silverberg, Messier, et al. 2010; Stamatovic et al. 2019). Recent studies have also linked a role for transcriptional regulation and differential expression of miRNAs to several processes in the ageing BBB (Inukai et al. 2012). Specifically, miRNAs have been shown to participate in cerebrovascular modulation and BBB dysfunction in vascular cognitive impairment, neuroinflammation and neurodegeneration (W. Liu et al. 2016; Toyama, Spin, and Tsao 2016). Thus, the potential role of miRNAs in the onset of healthy ageing is of interest to understand the underlying molecular mechanisms. Our sequencing results found that most of the upregulated genes in the female ageing BBB were associated with inflammatory and leukocyte adhesion pathways. These results are consistent with previous studies that reported a higher inflammatory state of the ageing brain in mice and humans (Donato et al. 2007; C. K. Lee et al. 2000; Mejias et al. 2018). Additionally, our results are also in line with gene expression analysis performed by Berchtold and colleagues that showed how differential expression patterns in the ageing human brain are sexually dimorphic with a greater immune activation in elderly women in comparison to age-matched men (Berchtold et al. 2008). Regarding miRNAs, a total of 9 miRNAs were differentially expressed in the female brain microvasculature during ageing, and among the predicted targets, several genes were related to response to stress and cell death pathways. Similarly, these results are consistent with available data that described processes of oxidative stress, endothelial inflammation and cell death during age-induced cerebrovascular dysfunction (Modrick et al. 2009; Park et al. 2007; Rodríguez-Mañas et al. 2009).

5.5 BBB alterations in ageing appear to be associated with miR-144-3p/*DNMT3A* deregulation

5.5.1 *MiR-144-3p/DNMT3A* deregulation induces changes in adhesion molecules but only *DNMT3A* downregulation promotes higher leukocyte adhesion to BECs

Among the several deregulated miRNAs identified in the aged microvessels, miR-144-3p was selected for further analysis. Previous studies have reported upregulation of miR-144 in the aged cortex of non-human primates and patients with AD (Persengiev et al. 2011). MiR-144-3p has been reported to promote neuroinflammation via microglia activation in intracerebral haemorrhage in mice (Yu et al. 2017), and to suppress anti-inflammatory proteins such as Nrf2 in male rat brain after ischaemia (Chu et al. 2019; Liu et al. 2014). Although no evidence is available regarding the role of miR-144-3p in BBB, it has been implicated in modulating TJ protein regulation in the blood-tumour barrier of human glioma, increasing permeability (Cai et al. 2015). These findings suggest a potential role of miR-144-3p in the age-related inflammation occurring in the female brain and BBB. In addition, target prediction analysis determined age-deregulated *Dnmt3a* as a predicted target of miR-144-3p, which is consistent with recent studies that have also predicted a reciprocal relationship between miR-144-3p and *DNMT3A* in women (Xu et al. 2020). In the present study, *Dnmt3a* mRNA and protein levels were found to be significantly downregulated in the ageing female cerebrovasculature. *Dnmt3a* is one of the DNA methyltransferases in mammals, which together with *Dnmt1* and *Dnmt3b* modulate DNA methylation. DNA methylation patterns have been shown to be significantly changed in several tissues of different species, including the brain (Bollati et al. 2009; Christensen et al. 2009; Heyn et al. 2012). Although not in ageing, deregulated DNA methylation has been related to changes in TJ proteins in mice (Stamatovic et al. 2019), and the overexpression of genes related to amyloid pathology in cultured murine BECs exposed to A β (Chen et al. 2009). Accordingly, DNMT expression is altered with ageing, which includes age-dependent downregulation of *DNMT3A* expression in neurons of the ageing human brain (Siegmond et al. 2007). These findings suggest that age-related changes occurring at gene expression level in the female mouse BBB might be related to *Dnmt3a* expression modulation.

Our analysis of a reciprocal relationship between miR-144-3p expression and *Dnmt3a* levels were confirmed in a human cell line (hCMEC/D3). As our sequencing results indicated

that inflammation and leukocyte migration pathways were increased in the aged female BBB, both leukocyte adhesion and paracellular permeability were assessed in the context of miR-144-3p/*DNMT3A* deregulation. No significant differences in paracellular permeability were observed after upregulating expression of miR-144-3p, thus experiments altering expression of *DNMT3A* were not performed. However, additional experiments to determine whether *DNMT3A* deregulation affects paracellular permeability, independently of miR-144-3p, would aid to understand the underlying mechanism of *DNMT3A* role in the ageing BBB. By contrast, deregulation of *DNMT3A*, although not miR-144-3p, was shown to alter leukocyte adhesion to hCMEC/D3 cells. Indeed, *DNMT3A* downregulation induces a significant increase of leukocyte adhesion, which suggests that at normal levels *DNMT3A* exerts an anti-inflammatory effect. Little information is available regarding the role of *DNMT3A* in leukocyte adhesion and BBB disruption. However, previous studies have reported that downregulation of another *Dnmt*, *Dnmt3b*, is linked to higher MMP activity in cultured murine BECs, leading to increased BBB permeability (Kalani, Kamat, and Tyagi 2015). Additionally, *Dnmt3a* loss leads to activation of inflammatory responses in mouse mast cells *in vivo* and *in vitro*, including higher reaction to stimuli and increased cytokine production (Leoni et al. 2017). Similarly, *Dnmt3a* loss in female transgenic mice has been related to inflammation and increased levels or pro-inflammatory cytokines, especially TNF- α , and upregulation of pro-inflammatory signalling genes such as NF- κ B (Jacquelin et al. 2018). The potential anti-inflammatory effect of *DNMT3A* is supported in our results by the observation that its deregulation is concomitant with upregulation of *ICAM-1* and *VCAM-1* adhesion molecules and the chemokine *CCL5(RANTES)* in hCMEC/D3 cells. Interestingly, *VCAM-1*, but not *ICAM-1* or *CCL5*, is upregulated when miR-144-3p is upregulated, even though miR-144-3p deregulation did not show any effect on leukocyte adhesion. The miR-144-3p effect on *VCAM-1* expression might be due to the indirect downregulation of *DNMT3A* mediated by miR-144-3p. It is well established that *ICAM-1* and *VCAM-1* are upregulated in human BECs under inflammatory conditions including exposure to pro-inflammatory cytokines (Stins et al. 1997), promoting adhesion and transmigration of leukocytes across the cerebrovasculature in the human brain (Nagai and Granger 2018; Supanc et al. 2011). Chemokines such as *CCL5* also contribute to leukocyte migration across the BBB in the context of inflammation, as reported in human BECs *in vitro* and in female EAE model mice *in vivo* (Quandt and Dorovini-Zis 2004; Dos Santos et al. 2005; Subileau et al. 2009). Despite the fact that little information is available regarding *ICAM-1/VCAM-1/DNMT3A*, and although is not exposed in the context of BBB but in a LPS-stimulated macrophage line, *Dnmt3a* inactivation in mice has been recently associated with

overexpression of Ccl5 among other chemokines and cytokines (Sano et al. 2018). Taken together, these findings suggest that DNMT3A plays an anti-inflammatory role in the BBB, and, since DNMT3A participates in *de novo* methylation, we can speculate that age-related depletion of DNMT3A expression levels might induce DNA methylation changes that, directly or indirectly, lead to overexpression of pro-inflammatory mediators (e.g. ICAM-1, VCAM-1 and CCL5) thereby contributing to leukocyte adhesion to the endothelial cells of the BBB.

5.5.2 Dnmt3a and Icam-1 protein expression are significantly deregulated in the ageing female cerebrovasculature

To determine whether the changes in expression of *ICAM-1* and *VCAM-1* observed in the *in vitro* experiments are also occurring in the ageing female mouse BBB, protein levels were assessed *ex vivo* by immunohistochemistry in the female brain cortex. Despite being significant, *CCL5* overexpression in the context of *DNMT3A* deregulation was small, therefore, we decided to focus on the adhesion molecules for this last set of experiments. *Ex vivo* analysis found that *Dnmt3a* was significantly decreased in the female cerebrovasculature with age. By contrast, although no changes were found at mRNA level, *Icam-1* protein was shown to be significantly decreased in the brain vessels of aged female mice, while *Vcam-1* was unchanged. These observations suggest a potential difference in *Dnmt3a*-mediated regulation of *Icam-1* and *Vcam-1*, since downregulation of *Dnmt3a* is concomitant with overexpression of *Icam-1*, but not *Vcam-1* in the female cerebrovasculature. In fact, *Dnmt3a* has been reported to participate in several processes including tumour suppression, hematopoietic cell differentiation, neurogenesis and inflammation (Jacquelin et al. 2018; Yang, Rau, and Goodell 2015), therefore, within the complex network of modulated genes, many other factors might be participating in the *Dnmt3a*-mediated regulation of adhesion molecules, resulting in a differential regulatory mechanism depending on *Icam-1* or *Vcam-1*. This theory of a differential regulatory effect mediated by *Dnmt3a* might be supported in our study by a higher increase in *ICAM-1* expression compared to *VCAM-1* when *DNMT3A* is silenced in hCMEC/D3 *in vitro*. However, additional experiments assessing the differences in DNA methylation in aged brain microvessels versus young brain microvessels would aid understanding whether *Icam-1* and *Vcam-1* expression is directly affected by *Dnmt3a* and if so, whether they are differentially demethylated.

The age-mediated upregulation of Icam-1 observed in the ageing female mouse brain microvessels is in contrast with previous studies which have reported that the process of ageing in the brain is associated with inflammation and loss of TJ proteins but not with leukocyte transmigration in female mice (Elahy et al. 2015). However, our results are in agreement with previous studies that reported a higher expression of Icam-1, but not Vcam-1, in BECs of aged male mice when exposed to inflammatory cytokines (Xu et al. 2010). These findings suggest a differential deregulation between Icam-1 and Vcam-1 in ageing, which might be due to Dnmt3a age-dependent deregulation and might influence leukocyte adhesion.

5.6 Limitations of the current studies and future work

Combining TEM techniques with 3D reconstruction gives a high-throughput profiling of capillary structural features. Due to its high resolution, this same technique has been used before to assess perivascular astrocytic coverage under pathological conditions (Mathiisen et al. 2010). The main caveat of this approach is that the process is very time consuming and therefore limits the number of vessels that can be analysed and restricts the analysis to certain brain cellular and/subcellular components. Therefore, even though some of the structural features assessed appear to have a trend towards change, generalization is not possible in some of these cases. Alternatively, other methods of serial 3D reconstructions include large-scale processing of previously stained brain sections in a quicker and more efficient way (Berlanga et al. 2011; Nizari et al. 2019). However, such approaches do not provide the higher resolution to discern ultrastructural features. To obtain a much more extensive profiling of structural changes in the ageing BBB with same accomplished resolution, a higher number of vessels should be analysed, although the amount of work that this requires should be considered.

The present study has reported regional ultrastructural differences between aged cortex and hippocampus, including a more inflammatory state or higher TJ tortuosity in cortical capillaries compared to hippocampal capillaries. Additional experiments including assessment of *in vivo* barrier permeability in cortex and hippocampus, or isolation of hippocampal microvessels to perform RNA sequencing as we did for cortical microvessel, would be useful to determine if the ultrastructural region-dependent differences that we observed have a real impact on BBB function. Also, further comparison of BBB changes between these and other

brain regions would help understanding the role of the structural changes observed in this study on BBB function and whether sex specificity is contributing to the regional differences.

Regarding our RNA sequencing analysis, the main limitation we encountered was the low RNA yield obtained in our first trials for single BEC isolation. Previous studies have reported higher yields of isolated microvessels, but they were isolating whole brain regions (e.g. cortex or hippocampus) and pooling them together, thus having enough starting material (Yousef et al. 2018). In contrast, we aimed to extract RNA from specific microvascular cells and therefore the amount of starting material was much lower. Additionally, other groups have managed to successfully isolate single cells from brain tissue with acceptable RNA yields using magnetic beads (Vanlandewijck et al. 2018). Unfortunately, this same approach did not work in our hands, which together with the restrictions in number of animals available made us select a more classical protocol for microvessel fragments isolation. As stated by Yousif and colleagues, one major handicap of this kind of isolation is the less purified final sample, which becomes a problem for experiments aiming to identify and locate specific genes or proteins in certain BBB components (Yousif et al. 2007). Which is even more complex when cell proportions change between groups, as observed for SMA- α and PDGFR- β in our data. Therefore, it is essential to take this into consideration when analysing sequencing results. Nevertheless, we considered all these limitations and managed to select an age-deregulated mRNA/miRNA pair expressed in BECs. However, studying in the same way the different cell types at the brain microvasculature would give a better insight of how the female BBB changes in ageing.

The predicted targeting relationship between miR-144-3p and *DNMT3A* has been partially confirmed by transfection of hCMEC/D3 in which both mRNA and miRNA changed in opposite directions. However, additional confirmation could be performed using 3'-UTR-luciferase assay and luciferase reporter vectors (Lee et al. 2007). Briefly, the protocol consists on transfecting these vectors, containing the potential target site that is also present in the mRNA of interest, into the cells and measuring luciferase activity. Luciferase activity will be reduced if the miRNA is actually binding to the target sequence, which suggests a targeting relationship (Hirata et al. 2012; Zhang et al. 2010). As mentioned above, *DNMT3A* deregulation, was shown to promote a proinflammatory phenotype in hCMEC/D3 cells, inducing an increase in leukocyte adhesion. Although we did not pursue paracellular permeability alteration after observing no significant change for miR-144-3p, leukocyte

adhesion experiments have shown that *DNMT3A* might be the one exerting a higher effect on BBB integrity. Therefore, assessing paracellular permeability in hCMEC/D3 in the context of *DNMT3A* deregulation would aid to better understand its role in age-mediated BBB dysfunction.

Also, in order to link *DNMT3A* deregulation back to female BBB dysfunction and sex differences in ageing, some extra experiments involving exposure to sex steroid hormones could be done. Indeed, previous studies have proved the anti-inflammatory effects of oestrogen in the brain and BBB protection (Duckles and Krause 2011; Ghisletti et al. 2005; Vegeto et al. 2003), as well as the inflammatory detrimental age-related effects in cerebrovascular function related to oestrogen loss during and after reproductive senescence (Deer and Stallone 2016; Henderson and Lobo 2012; Sunday et al. 2007). Similarly, results obtained by Berchtold and colleagues suggest that upregulation of inflammatory genes in the female brain are not solely driven by age itself, but may be related to oestrogen withdrawal in menopause (Berchtold et al. 2008). Thus, a logical next step would be to repeat the same set up *in vitro*, but including several concentrations of oestrogen in the culture in order to assess a possible hormonal effect in miR-*DNMT3A* deregulation.

Our results showed that *DNMT3A* silencing is concomitant with upregulation of both *ICAM-1* and *VCAM-1* in human BECs. However, no changes in mRNA or protein levels were observed for Vcam-1 in the brain of ageing female mice, while Icam-1 was significantly upregulated at protein level only. Additional experiments could assess changes in DNA methylation between aged and young cortical capillaries, to determine whether Icam-1 and Vcam-1 are differentially modulated at the methylation level. Similarly, possible post-translational modifications in Icam-1 protein could be assessed, in order to understand changes at the protein level while mRNA levels remain unchanged. Lastly, a final limitation of the study is that the described effect of *DNMT3A* deregulation on leukocyte adhesion to brain microvascular endothelial cells has only been assessed by *in vitro* techniques. In light of the existing controversy regarding leukocyte adhesion in the healthy ageing brain (Elahy et al. 2015; Miguel-Hidalgo et al. 2007), *in vivo* experiments would be useful to assess whether the same conditions are translated into the complex and much richer environment of the brain.

In any case, we have ended up selecting a couple of the several candidates that were shown to be deregulated in ageing. The sequencing dataset obtained from this project will work

as a starting point to assess different mechanisms happening at the female ageing BBB in the future.

5.7 Conclusions

The novel observations generated from this PhD project are that:

1. Healthy ageing induces structural changes in the female BBB that are more prominent in cortical capillaries than in hippocampal capillaries. Among the structural age-related modifications, we found higher number and volume of BEC surface pseudopods, larger volume of pericyte mitochondria and increased coverage of pericytic projections over brain endothelium in aged capillaries compared to vessels from young female mice.
2. MiR-144-3p, previously described to be upregulated in the ageing brain in neurons, is also upregulated at the level of brain microvasculature during ageing.
3. MiR-144-3p appears to negatively regulate *Dnmt3a*. Moreover, both are changed in opposite directions during ageing. *Dnmt3a* protein levels are also significantly downregulated in the mouse cerebrovasculature.
4. *In vitro* induced deregulation of miR-144-3p does not affect paracellular permeability of brain endothelial cells.
5. *In vitro* induced deregulation of *DNMT3A*, but not miR-144-3p, promotes alterations in leukocyte adhesion to brain endothelial monolayer. Indeed, when *DNMT3A* is downregulated, leukocyte adhesion is significantly increased.
6. *ICAM-1*, *VCAM-1* and *CCL5* expression levels are deregulated in hCMEC/D3 cells after miR-144-3p/*DNMT3A* deregulation. *In vitro* experiments showed that only *VCAM-1* is increased when miR-144-3p is upregulated. *DNMT3A* silencing promotes significant upregulation of both adhesion molecules and *CCL5 in vitro*,

whereas only *Icam-1* protein expression appears to increase in the ageing cerebrovasculature from female mouse tissue.

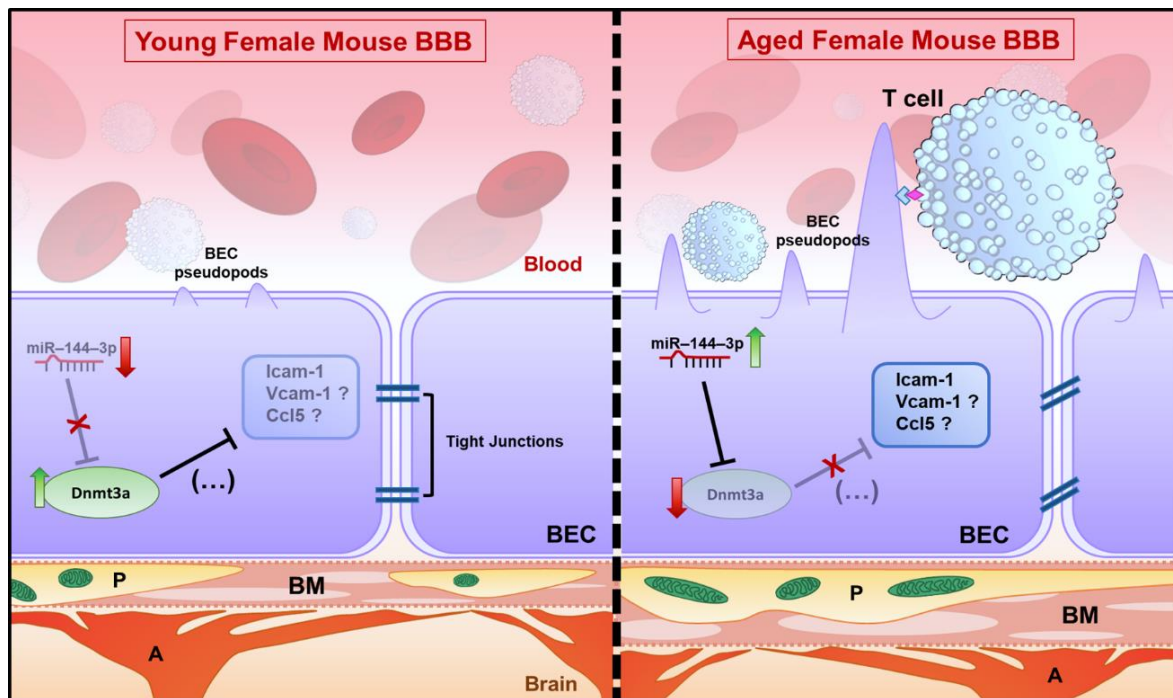


Figure 44. Age-related ultrastructural and gene expression changes in the female mouse BBB: A summary.

In the present study, brain cortical capillaries were shown to be more affected than hippocampal capillaries during normal ageing. The ultrastructural analysis showed a significant increase in BM thickness, higher number and volume of BEC pseudopods, increased pericyte mitochondrial volume and coverage, and higher TJ tortuosity. MACE-Seq results showed most of the upregulated genes to be related to inflammatory pathways. MiR-144-3p and *Dnmt3a* were shown to have an inverse correlation in the ageing female mouse BBB and in human BECs (hCMEC/D3). However, *Dnmt3a* appears to have a most important effect in inducing the pro-inflammatory effect on BECs, independently of miR-144-3p. Indeed, *DNMT3A* downregulation increases Jurkat T cell adhesion to hCMEC/D3 monolayers and promotes upregulation of *ICAM-1*, *VCAM-1* and *CCL5*, at the mRNA level. By contrast, only *Icam-1* was proved to be downregulated at the protein level in the aged female mouse microvessels. P: pericyte, A: astrocyte. (Diagram by Eduardo Frías Anaya).

In conclusion, healthy ageing appears to affect the structure of female brain microvasculature in a differential way depending on the region, with more prominent alterations in cortical capillaries (**Figure 44**). Such structural differences may be associated with increased vessel stiffness, higher number and volume of BEC surface projections, changed pericyte coverage and mitochondrial dynamics and altered TJ tortuosity. At the molecular level, the female BBB in the cortex is mostly inflammatory active during ageing, with several deregulated mRNAs and miRNAs among which miR-144-3p appears to mediate expression of

DNMT3A, which in addition appears to promote leukocyte adhesion onto brain endothelium when downregulated. The implications of this work suggest a low-grade but increasing cerebrovascular inflammation during ageing that may have further consequences on female BBB and brain function. Collectively, these alterations may also contribute to the specific sex differences observed regarding incidence and progression of cerebrovascular diseases between males and females, as well as the development of certain age-induced neurodegenerative disorders and vasculopathies that appear to be more prevalent in post-menopausal women.

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Appendix

Table 5. List of differentially expressed genes (mRNAs) between young and aged female mouse microvessels as analysed by MACE-Seq analysis.

Negative values mean downregulation in young microvessels with respect to aged microvessels (increased in ageing), whereas positive values mean upregulation in young microvessels with respect to aged microvessels (decreased in ageing).

Gene symbol	log2FoldChange (Young vs Aged)	P-value
Spc24	-4,988975934	0,00077756
Fpr2	-4,877932973	0,002410934
Ms4a3	-4,140862536	0,018412749
4930519F09Rik	-4,140862536	0,020965862
C030034I22Rik	-4,140862536	0,021435008
Fgf21	-4,140862536	0,03610812
Pilrb1	-4,041588956	0,03119602
BC043934	-3,934371332	0,032061357
Gm13536	-3,823500359	0,002716675
Itga2	-3,819196535	0,045052234
Epha1	-3,697939689	0,004624725
Izumo1	-3,486586084	0,013743093
Clec4d	-3,326097067	0,0214937
Ubash3a	-3,238865483	0,023325952
Lancl3	-3,238865483	0,029491735
Ms4a4b	-3,175748544	0,007557917
Ifitm6	-3,163730509	0,033618673
Ifi204	-3,145493985	0,035622085
Aspg	-3,145493985	0,038887063
Adgrf4	-3,111431719	0,010086853
Gm8276	-3,111431719	0,018736733
Ccnb2	-3,044487731	0,014710657
Iglc3	-3,003241492	0,004401039
Glipr1	-2,988253069	7,73E-06
Prg2	-2,974089042	0,000638213
Il20rb	-2,97389245	0,014860775
Gssos2	-2,97389245	0,01919566
Saa2	-2,939002781	0,008114792
Mki67	-2,939002781	0,009533309
Ppbp	-2,870079405	0,007863125
AI662270	-2,86149211	0,001985824
Gm44805	-2,823500359	0,007869773
Tfap2a	-2,821845665	0,020986294
Arhgap27os1	-2,762256513	0,021194954

Oas1b	-2,697939689	0,011163393
Asf1b	-2,697939689	0,011586757
2310040G07Rik	-2,697939689	0,013414965
Cd3d	-2,697939689	0,014113233
Pycr1	-2,697939689	0,021659002
Guca1a	-2,652357513	0,040423026
Ctsk	-2,630995701	0,018706188
Galnt4	-2,630995701	0,018974841
Map4k1	-2,560400419	0,036949694
Hist1h1e	-2,560018711	0,001968586
Cdk1	-2,559708847	0,016934246
Ltb4r1	-2,558986015	0,007655356
Plac8	-2,524625807	6,06E-07
Cd2	-2,500644784	0,012061216
Pgm3	-2,384917511	0,005896279
Spn	-2,375262831	0,001420609
AC164087.2	-2,352494811	0,029397125
Ccl5	-2,316911797	1,64E-08
Ly9	-2,308140126	0,031597873
Cks2	-2,308140126	0,037292434
Poli	-2,23792212	0,025237668
Gm35040	-2,237741437	0,003806356
Hist1h2ad	-2,236992389	0,04590354
Tmem255a	-2,185589915	0,04194061
Tlr13	-2,175748544	0,022993016
Retnlg	-2,167164849	0,033753732
Fam227b	-2,155656726	0,009107279
Ptprcap	-2,155656726	0,019608343
Ube2c	-2,112700133	0,021643192
Acp5	-2,08112824	0,001461106
Nphp4	-2,044780331	0,028512995
Clec7a	-2,031335923	0,005470133
Fam81b	-2,022494638	0,020064873
Cd300e	-2,01548289	0,038508941
Cst7	-2,003241492	0,016070484
Hist1h2ao	-1,996965189	0,000614799
Acot10	-1,992448126	0,028118404
Ear2	-1,988146805	0,016407645
Fcgr4	-1,950353136	0,001231504
Gm43362	-1,926270576	0,024435185
Hist1h2ap	-1,921466597	0,000362065
Atad2	-1,908182905	0,011286865
Serpina3n	-1,900850642	0,024263718
Tmem132e	-1,88992222	0,048310184
Top2a	-1,881405165	0,041242394
Gm26510	-1,870079405	0,021019074
Gm14450	-1,866243793	0,01260157

H2-Q5	-1,862264637	0,02631063
Itgax	-1,8232872	0,025432917
Haus6	-1,823167041	0,049115603
Tmem71	-1,822455447	0,010631605
Ccr2	-1,822341102	0,049757788
Mmp9	-1,799828185	0,002634193
Mrps36-ps1	-1,757037877	0,009159888
Itgal	-1,752579543	0,013401165
Sp6	-1,740361625	0,017755987
Slco5a1	-1,723084013	0,033519504
Gm13056	-1,723084013	0,041215304
Dsn1	-1,71212408	0,048787151
Gm45113	-1,71212408	0,04919017
Ccl8	-1,711608924	0,012858937
Alas2	-1,702297161	1,54E-06
Rac2	-1,702050237	0,000148153
Lrriq1	-1,670751808	0,044844313
AI854703	-1,664276207	0,021862224
Cybb	-1,659404611	0,000670079
Milr1	-1,636857644	0,043683944
Tirap	-1,630415956	0,013954666
Lgals3	-1,619547544	2,83E-05
1700086O06Rik	-1,616298424	0,014750471
Gm28153	-1,611273778	0,003675271
Gm15397	-1,607199537	0,042967967
Olfir78	-1,560018711	0,03939871
Snx33	-1,544543611	0,011649498
Cplx3	-1,544543611	0,02753474
Myo1g	-1,527162908	0,016593065
Hbb-bt	-1,523868436	0,000528235
Phf11d	-1,518079599	0,001550341
Slc6a12	-1,510975476	0,047286661
Pygl	-1,500808606	0,01958119
Dph5	-1,500754	0,03134274
Cenpa	-1,495843858	0,008552917
Ttc5	-1,493900454	0,025259639
Hbb-bs	-1,486395583	0,002862353
Clec4a1	-1,481796701	0,03594314
Gfap	-1,477869327	3,73E-05
Tarsl2	-1,472315775	0,028492959
Zfp612	-1,472315775	0,033758194
Gm9333	-1,47034782	0,014870198
Hba-a1	-1,462109794	0,001334747
Mettl13	-1,449458622	0,022179062
Hba-a2	-1,440744231	0,001308799
Ighg2c	-1,435684045	0,032016019
Tbccd1	-1,425174323	0,0288716

Ptprc	-1,424240755	0,005341541
Arhgef19	-1,414987097	0,028502239
Topors	-1,413663177	0,010328667
E230029C05Rik	-1,403337358	0,002275186
Dock5	-1,400223425	0,033241307
Pdcd7	-1,386892666	0,040307623
Gm9946	-1,383798807	0,003999827
Tram2	-1,382335917	0,026587593
Gm37893	-1,382335917	0,043551849
Lyrn7	-1,380827966	0,016756963
Lyz1	-1,37522709	0,019489138
S100a4	-1,373698123	6,57E-05
Tsacc	-1,367456435	0,048756632
Fgr	-1,359718843	0,049350378
A630001G21Rik	-1,348678097	0,014621371
Zfp688	-1,337476516	0,03120206
Plbd1	-1,329167728	0,032395936
Dapp1	-1,328077012	0,009334873
Tmem45a	-1,318857542	0,017454673
Runx1	-1,315630586	0,046978043
Fam118a	-1,313136345	0,000154631
Proca1	-1,308140126	0,041847081
Lyz2	-1,299630648	5,20E-06
Prkaa2	-1,29682898	0,028149581
Oasl1	-1,288557877	0,024160072
C3	-1,284145685	0,003312639
Crnk11	-1,281000257	0,009219948
Calhm2	-1,272651907	0,004790181
Spsb1	-1,259681835	0,014417298
Nexn	-1,258390178	0,010756861
Ppp1r32	-1,258390178	0,011943296
1700018A04Rik	-1,255324291	0,004674165
Gm10125	-1,237843599	0,008290379
1110002L01Rik	-1,237843599	0,047663351
Ankfn1	-1,220467334	0,00878274
Clcn2	-1,212768002	0,029856942
Usp39	-1,211993291	0,032630614
Fzd8	-1,211993291	0,048418578
5430405H02Rik	-1,210817108	0,04349332
Pus7	-1,196151504	0,021434225
C4b	-1,188979596	0,003635471
Wdr46	-1,181187479	0,028124293
Elp4	-1,17898405	0,005919335
Dok3	-1,174833082	0,019175011
Amy1	-1,167396261	0,010016094
Tapt1	-1,163730509	0,036818897
Extl1	-1,153777815	0,01396532

Mmgt2	-1,144798319	0,029604659
Hace1	-1,141631021	0,043887952
Dpf1	-1,139315576	0,02347733
Ifi30	-1,135270441	0,023141309
Fmod	-1,134348643	0,011291303
1700001L19Rik	-1,128242291	0,018078939
Lsp1	-1,108541735	0,003446112
Ccdc85b	-1,10287651	0,013993436
Oma1	-1,09704637	0,018008585
Ifi203	-1,090353908	0,027000323
Dcaf15	-1,090353908	0,036597698
Gm6030	-1,088214682	0,020011755
Isg20	-1,08274722	0,001818727
Pla1a	-1,078377012	0,019256084
Elov14	-1,078377012	0,024607258
Gm24447	-1,074221329	0,03416316
Rdh14	-1,063791288	0,028585945
Tbc1d23	-1,058076264	0,00990261
Trim12c	-1,058076264	0,021675791
Ccnc	-1,052767879	0,027742309
Foxp4	-1,052278986	0,026867029
Igf2bp3	-1,051478535	0,042305305
Zfp275	-1,050721125	0,042029612
Gxylt1	-1,049822888	0,043176185
Syde1	-1,047969345	0,005504716
Bet1	-1,045290898	0,014526372
Brd1	-1,040946131	0,021030379
Incenp	-1,040402354	0,034006133
Vav2	-1,039862467	0,031806062
Adamts2	-1,026451774	0,049374897
Ccl6	-1,016866467	0,004520333
Stx2	-1,015371325	0,04045781
Nos1	-1,012270403	0,000989768
Plekhh2	-1,005627474	0,0464297
Coa7	-1,005627474	0,048776447
Dnmt3a	1,000071005	0,030159302
Adh1	1,004595794	0,025677712
Bambi	1,028514975	0,029726779
Ednrb	1,042248037	0,042792592
Gem	1,051629924	0,047741874
5730480H06Rik	1,055161215	0,045395654
Rbm4b	1,068693501	0,045879129
Uevld	1,070157517	0,040951108
Phyhipl	1,070831771	0,00123077
Hes5	1,084105476	0,014415573
Csrnp1	1,087956155	0,004279508
Il18	1,103226805	0,001221479

Slc10a1	1,112843318	0,040763673
Tatdn3	1,12109399	0,035938253
Rab30	1,1247506	0,038422031
6330403L08Rik	1,14692595	0,049286867
Col8a2	1,151122905	0,035196858
Dcum1d3	1,172405991	0,001952102
Gm20532	1,177187357	0,019724056
Pot1a	1,197043043	0,010125899
F5	1,221629929	0,028249414
Tubd1	1,242121892	0,023865064
Ppp4r1	1,251133133	0,000266671
2610306M01Rik	1,273221866	0,016926541
Rslcan18	1,280149972	0,011192001
Cd33	1,282980794	0,00899663
Cmc4	1,288133521	0,005458346
Gm17208	1,300577916	0,010575167
Cox15	1,306577677	0,0064727
Gan	1,308086004	0,010335639
Slc29a4	1,316758463	0,01327616
1500026H17Rik	1,318501154	0,018530176
Zfp426	1,320354341	0,039254838
Ccdc112	1,322950084	0,010730855
3110002H16Rik	1,347080537	0,025952442
Mblac2	1,347093961	0,008750624
Gm24305	1,34714667	0,02322239
Gtf2ird2	1,372194498	0,012793825
Apoc1	1,380799321	0,033486173
Rhoh	1,386502743	0,0318404
B4galnt4	1,390341548	0,004935684
Rinl	1,4097856	0,046271885
Tada2b	1,45419797	0,018362363
Chst10	1,45419797	0,02281032
Rasl11a	1,468358241	0,008216168
Cort	1,498795777	0,028087469
Baiap2l1	1,512265733	0,005527313
Xkr8	1,512391038	0,047174844
B230312C02Rik	1,521272026	0,019790324
Jmjd4	1,521272026	0,024360023
Adora2b	1,550720095	0,028222221
Wdr86	1,550720095	0,036802548
Slc4a5	1,569372767	0,048819494
Tmem267	1,583213991	0,005508968
Gm15432	1,592515895	0,046969837
Mapk7	1,610252316	0,005400341
Zfp2	1,682487571	0,003908275
Gm33178	1,761907459	0,047183432
Pigo	1,762169552	0,004012306

1700029J07Rik	1,861443132	0,000877661
Cideb	1,877325234	0,0331538
Kl	1,926060726	0,044202038
Gm20521	1,955210813	0,016869259
Slc24a5	1,955210813	0,025611775
Clic6	1,980665329	0,028650906
Sec31b	2,025728289	0,048043023
Steap1	2,04262252	0,027590966
Fsd1l	2,083758278	0,005492359
Gm23238	2,203001067	0,004486706
Col9a3	2,233344973	0,031209581
Stk32c	2,248010315	0,020343858
AC162376.3	2,285301419	0,027411354
Gm5555	2,285301419	0,030712401
A630014C17Rik	2,285301419	0,033815304
Sgca	2,346869959	0,007174756
AC122273.2	2,346869959	0,021070594
Cblc	2,500338537	0,033257846
Gm16194	2,500338537	0,036629322
Ppp1r42	2,500338537	0,049031974
Gm13111	2,6199151	0,006523234
Chdh	2,637757769	0,028470264
Cldn2	2,668746538	0,00222697
Rbp7	2,701649864	0,019387343
Gm27032	2,932090015	0,028770829
Gm45606	3,009794301	0,027271098
AC153144.3	3,083990092	0,018839692
4731419I09Rik	3,083990092	0,026080383
Gm2464	3,5730884	0,047351008
Gm9768	3,672889007	0,034587461
Hgfac	3,672889007	0,035560992
Gm25890	3,672889007	0,045378423
Nppc	3,853352169	0,025599156
Gm7935	3,93602643	0,029266817
Stc1	4,013730716	0,016369389