The role of PDGF-B in brain blood vessels

KHAYRUN NAHAR
The development of blood vessels is dependent on several molecular cues to form properly. A functional PDGF-B/PDGFR-b signaling is paramount for the investment of mural cells, that provide with support, to the developing vasculature. Mutations in $\text{PDGFB}$ and $\text{PDGFRB}$ are linked to PFBC, an age-dependent neurodegenerative condition manifested by vessel associated calcifications in the brain. The overall aim of the work presented in here was to investigate PFBC related calcifications and analyze the effects of impaired PDGF-B/PDGFR-b signaling on the formation of brain calcifications in different mouse models.

In paper I, we functionally analyzed PFBC-related $\text{PDGFB}$ and $\text{PDGFRB}$ mutations in vitro. While all $\text{PDGFB}$ mutations lead to abolished protein function, $\text{PDGFRB}$ mutations have more diverse consequences. We also show that reduced $\text{Pdgfb}$ and $\text{Pdgfrb}$ levels are insufficient for the formation of brain calcifications in several mouse strains. Moreover, region-specific susceptibility factors seem to reside in PFBC pathogenesis that are distinct from pericyte coverage and BBB deficiency.

In paper II, we described the molecular composition and cellular association of calcified nodules that develop in two mouse models of PFBC, $\text{Pdgfb}^{\text{ret/ret}}$ and $\text{Slc20a2}^{-/-}$ mice. We show that the nodules are composed of pro- and anti-mineralization proteins and that they are in direct association with astrocytes and microglia.

In paper III, we analyzed the effects of EC-specific ablation of PDGF-B in adult brain vasculature. We report a substantial decrease of pericyte coverage and altered VSMC morphology and that this phenotype is inadequate to trigger the formation of calcifications or affect BBB integrity.

The aim of paper IV was to molecularly define the adult mouse brain vasculature by taking advantage of the scRNaseq technique. Here, we describe a gradual change in expression profile along the arteriovenous axis: ECs present a continuum along the axis while mural cell expression profile is punctuated.

In summary, this thesis present detailed description of calcifications formed in mouse models of PFBC and address the role of impaired PDGF-B/PDGFR-b signaling for the formation of nodules in mice. Furthermore, the scRNaseq analysis performed on healthy adult brain vasculature has paved the way for future analysis in mouse models of PFBC.

Keywords: PFBC, PDGFB, PDGFRB, Brain vasculature, Mural cells

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“I’m tired of reading about the achievements of better men”

~Samwell Tarly
(GoT, S07E05)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II  Nahar, K., Lebouvier, T., Mäe, MA., Konzer, A., Bergquist, J., Zarb, Y., Johansson, B., Vanlandewijck, M., Betsholtz, C. Astrocyte – microglial association and matrix deposition define common pathogenic events in primary familial brain calcification. *Manuscript*

III  Nahar, K., Bertuzzi, G., Vanlandewijck, M., Keller, A., Betsholtz, C., Mäe, MA. Endothelium-derived PDGF-B is essential for adult brain pericyte and vascular smooth muscle cell maintenance. *Manuscript*


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Other papers by the author


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<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
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<td>ABC</td>
<td>ATP-binding cassette</td>
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<td>AJ</td>
<td>Adherens junction</td>
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<td>AQP4</td>
<td>Aquaporin 4</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>BM</td>
<td>Basement membrane</td>
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<td>CBF</td>
<td>Cerebral blood flow</td>
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<td>COL1A1</td>
<td>Collagen 1 alpha 1</td>
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<td>CT</td>
<td>Computed tomography</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>DFSP</td>
<td>Dermatofibrosarcoma protuberans</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<td>GLUT1</td>
<td>Glucose transporter 1</td>
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<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
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<td>IBGC</td>
<td>Idiopathic basal ganglia calcifications</td>
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<td>IM</td>
<td>Infantile myofibromatosis</td>
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<td>KO</td>
<td>Knockout</td>
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<td>LoF</td>
<td>Loss-of-function</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>MYORG</td>
<td>Myogenesis regulating glycosidase</td>
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<td>NVU</td>
<td>Neurovascular unit</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<td>PFBC</td>
<td>Primary familial brain calcification</td>
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<td>Type III sodium-dependent phosphate transporter 2</td>
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<td>Solute carrier</td>
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<td>Solute carrier family 20 member 2</td>
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<td>SWS</td>
<td>Sturge-Weber syndrome</td>
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<td>TJ</td>
<td>Tight junction</td>
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<td>TS</td>
<td>Tuberous sclerosis</td>
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<td>VE-cadherin</td>
<td>Vascular endothelial-cadherin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor-A</td>
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<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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<tr>
<td>XPR1</td>
<td>Xenotropic and polytropic retrovirus receptor 1</td>
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The vascular network

The blood vascular network, also known as the cardiovascular system, is a circulatory system essential to sustain physiological functions of all other organs and cells in the vertebrate organisms. In a simplified view, the blood vasculature can schematically be visualized as vessels of different sizes, giving rise to a closed and fluid-filled tubular network driven by the contractions of a muscular pump, the heart. Some of the main functions of blood vessels are to transport necessary substances (e.g. oxygen, nutrients) to the organs to maintain normal physiological functions, regulate body heat, filtration and remove waste products and deliver immune cells to their final destination (Mohanhan-Earley et al., 2013).

Blood vessels are arranged in a hierarchical manner, comprised of pressurized large arteries that carry oxygenated blood from the heart and feed into smaller arterioles (Potente and Mäkinen, 2017). Arterioles ultimately branch into a network of small sized capillaries and it is here, at the surface of the capillary network, where the exchange of nutrients and gases between the blood stream and the surrounding tissue occur. In the majority of organs, non-oxygenated blood then flows back through venules and larger veins in order to get rid of waste products and become re-oxygenated in the lungs, eventually closing the loop in the heart and re-circulating the blood.

The innermost lining of blood vessels, the endothelium, is composed of endothelial cells (ECs) that are enwrapped by a basement membrane (BM) and also supported by mural cells. Mural cell is a collective term for vascular smooth muscle cells (VSMCs) that wrap ECs of larger caliber vessels, and pericytes that cover ECs on capillaries and venules (Betsholtz, 2018).

Every organ has its own demands and requirements to fulfill their specific physiological functions. Accordingly, this has resulted in the adaptation and specialization of different vascular beds in different organs leading to vascular heterogeneity/vascular organotypicity (Betsholtz, 2018; Potente and Mäkinen, 2017). Heterogeneity can also be found within the same vascular bed of an organ. For instance, zonation of liver ECs displaying a gradual change in marker expression according to their anatomical location and functional properties exists (Halpern et al., 2018).

Gaining more insight into how the vascular system and all its components function has been under the scope of intense research for a very long time (and still is). In fact, a dysfunctional vascular system has been implicated in several human conditions, ranging from cardio- and cerebrovascular diseases to
cancer. Although our knowledge of how such an intricate system, in both health and disease, is expanding it wouldn’t be excessive to claim that we have only just began to understand the broadness of its complexity.

The cerebrovascular system

The human brain receives up to 20% of all the cardiac output, making it one of the highest demanding organs when it comes to total energy consumptions (Andreone et al., 2015). A rough estimation of the total capillary length in the average human brain is an astonishing 644 km and can cover a surface area of up to 20 m² (Begley and Brightman, 2003)! To put things into perspective, if cerebral blood flow (CBF) comes to a halt the brain stops to function within seconds and neuronal damage can occur in just a few minutes. Disruption of CBF, and the subsequent perturbation in substance delivery and removal of by-products to and from brain cells respectively, has been implicated in neurodegenerative diseases such as Alzheimer’s disease (Zlokovic, 2008).

Arterial blood is supplied to the brain through the internal carotid artery and distal vertebral artery that forms the ring structure of the circle of Willis (Prince and Ahn, 2013). Large arteries are spread out on the surface of the subarachnoid space and divide into smaller pial arteries and arterioles that subsequently penetrate inwards and enter the brain tissue (Girouard and Iadecola, 2006) (Figure 1A). These vessels consist of ECs, VSMCs and astrocyte end feet. As the arteries and arterioles gradually become smaller they lose the smooth muscle cell layer and turn into capillaries covered by pericytes and astrocyte end feet. In addition, brain blood vessels are in contact with nerve endings and other resident cells of the brain (see below, section on NVU) (Figure 1B). It has been demonstrated that the capillary density in rat brain varies between the grey and white matter, reflecting local activity and metabolic demands (Borowsky and Collins, 1989).

Properties of the BBB

The observation that the central nervous system (CNS) regulate the passage of molecules between the blood and different CNS tissues was made already in 1885 by Paul Ehrlich (Ehrlich, 1885). He performed experiments with subcutaneous injections of water-soluble dyes and noticed that it stained almost all organs except for the brain and spinal cord. Subsequent studies performed in similar manners and with electron microscopy studies confirmed the existence of CNS barriers in the retina (Cunha-Vaz et al., 1966; PALM, 1947) and brain (Reese and Karnovsky, 1967). Regulated passage of substances have also been described for blood-nerve barrier, blood-spinal cord barrier and
blood-cerebrospinal fluid (CSF) barrier, encompassing different molecular signatures and functions (Reinhold and Rittner, 2017).

Figure 1. (A) Overview of the cerebral vasculature. Larger pial arteries of the subarachnoid space penetrate into the brain tissue and subsequently become smaller sized blood vessels. Dashed insert is magnified in (B) where the transition from larger artery and arteriole to smaller capillaries is illustrated. There is shift in associated cell types and mural cell coverage along the vascular tree.

Within the brain parenchyma, neurons signal through chemical and electrical cues and require a homoeostatic microenvironment that is favorable for their communication. Maintenance of a homoeostatic microenvironment and protection against neurotoxic insult is fundamental and obtained by the blood-
The brain barrier (BBB), which is formed by specialized capillary ECs that regulate the transport of molecules between the blood and brain. These ECs are tightly connected to one another by adherens junction (AJ) and tight junction (TJ) proteins (Dejana et al., 2009; Reese and Karnovsky, 1967). Functionally, AJs are involved in the initiation of cell-cell contacts which provides the tissue with structural support, while TJs regulate the paracellular transport of solutes. Vascular endothelial cadherin (VE-Cadherin) is a transmembrane AJ that spans the cleft between adjacent cells and is connected to intracellular actin cytoskeleton proteins. Occludins and claudins are transmembrane TJ proteins that also span the extracellular space between adjacent cells (Furuse et al., 1993; Morita et al., 1999). In addition, TJs are connected to the actin cytoskeleton via regulatory scaffolding cytoplasmic proteins such as zonula occludens proteins. Among the TJ proteins, claudin-5 is expressed by ECs at the BBB (Liebner et al., 2000) and for long, it was believed that claudin-3 is also expressed there. However, it was recently demonstrated that ECs at the BBB lack expression of claudin-3 in mice (Castro Dias et al., 2019).

The properties of the tight barrier require alternative ways of delivering essential molecules to brain cells and also to rapidly remove toxic substances from the brain tissue. Lipid-soluble molecules can cross the barrier by passive diffusion through complex physiochemical modes while the transport of gases such as oxygen and carbon dioxide occur via passive diffusion that is dependent on blood flow (Abbott et al., 2010). Furthermore, ECs at the BBB are equipped with transmembrane influx and efflux proteins that selectively allows for the passage of molecules. One such influx protein that is highly expressed by brain ECs is the glucose transporter 1 (GLUT1) that belongs to the solute carrier (SLC) family of transporters. This transporter is critical to accommodate for the high glucose requirement of the brain (Brockmann, 2009; Crone, 1965) and the transport of lipids across the BBB is facilitated by the lipid transporter MFSD2A (Nguyen et al., 2014). The ATP-binding cassette (ABC) transporters consist of a large family of efflux proteins whose main functions are to actively pump out lipid-soluble compounds out of the brain parenchyma (Abbott et al., 2010).

Another route for macro-molecules across the endothelium of the BBB is transcytosis, the transport of substances in vesicles through endocytosis from the luminal to abluminal side of the vasculature (Nag, 2011). There are two types of transcytosis, receptor-mediated and adsorptive-mediated. An example of receptor-mediated transcytosis is the uptake of iron-containing transferrin by the Transferrin receptor from blood to brain (Jefferies et al., 1984; Moos and Morgan, 2000). Caveolae-facilitated uptake of positively charged molecules, such as cationic lipids and albumin, are internalized by adsorptive-mediated endocytosis (Pulgar, 2018).
The neurovascular unit (NVU)

The intimate cell-cell interactions between the components of the neurovascular unit (NVU) is essential to maintain the properties of the BBB. The functional units of the NVU (other than ECs) are mural cells, astrocyte end feet, microglia, BM and nerve endings (Figure 2). The constituents of the NVU contribute to the regulation and coupling of local neuronal requirements to changes in CBF (Girouard and Iadecola, 2006). Short descriptions of some of the NVU components are listed below.

**Mural cells:** the distinction between the two mural cell types, pericytes and VSMCs, has mainly focused on their morphology and anatomical locations. They share many of the gene expression in mice (e.g. *Pdgfrb*, *Cspg4*, *Des* and *Anpep*) and are believed to derive from the same cell lineage (Armulik et al., 2005). VSMCs constitute a separate layer in the vessel wall and tightly wrap the vasculature with alpha-smooth muscle actin (α-SMA) fibers on arteries and arterioles. In contrast, pericytes do not express α-SMA, share their BM with ECs and extend long primary processes with secondary thin processes along smaller vessels. Brain pericytes have been attributed to maintain and regulate transport across the BBB (Armulik et al., 2010; Daneman et al., 2010). The identity of a pericyte is still poorly understood and their proposed functional roles are various - contractile abilities and regulation of vascular tone (Hall et al., 2014; Hill et al., 2015), stem cell properties (Crisan et al., 2008; Guimarães-Camboa et al., 2017), their role in pathological scarring (Görritz et al., 2011; Soderblom et al., 2013) and the presence of different subpopulations have been reported in the literature (Görritz et al., 2011). However, it is worth mentioning that many of the assigned pericyte properties have sparked controversy in the field with publications of conflicting data.

• **Astrocytes:** these are the most abundant glial cells in the adult CNS and acquire a spectrum of functions ranging from uptake and recycling of neurotransmitters to maintaining the properties of the BBB (Rothstein et al., 1996). Regional specific differences, where astrocytes perform distinct functions, have been reported to give rise to different subsets of cell identities that gradually changes with age (Clarke et al., 2018). At the NVU, astrocytes are located on the abluminal side of blood vessels through their slender “foot processes” (also called astrocyte end feet) and cover the entire cerebral vasculature (Stewart and Wiley, 1981). These processes are highly enriched with water channels (e.g. aquaporin 4 (AQP4)) and plays a fundamental role in controlling water homeostasis at the barrier (Jung et al., 1994). In addition, astrocyte end feet are believed to regulate CBF through vessel constriction and dilation, thereby adjusting CBF to local neuronal activity and demand (Attwell et al., 2010).
Figure 2. Cellular and basement membrane contribution to the neurovascular unit (NVU) complex. The NVU is an extension of the blood-brain barrier (BBB) and the intimate cell-cell interaction between the components of the complex maintains the properties of the BBB.

- **Microglia:** often referred to as the resident immune cells of the CNS. Resting microglia are equipped with long, thin processes radiating out from a small cell body (Helmut et al., 2011). During pathological situations such as in Alzheimer’s disease, microglial gets activated, change appearance to an amoeboid-like structure and become phagocytotic (Griciuc et al., 2013). The changes in morphology and function in activated microglia is accompanied by the release of cytokines and changes in surface antigen expression (Berg et al., 2012).

- **Basement membrane:** a structural support and integral part of the extracellular matrix that connects and holds the separate building blocks of the NVU together. The BM is mainly comprised of proteoglycans, different collagen isoforms, laminins and nidogens (Engelhardt and Sorokin, 2009;
Sorokin et al., 1994). These components are produced and maintained by endothelial cells, pericytes and astrocyte end feet (Gautam et al., 2016; Sorokin et al., 1994; Y. Yao et al., 2014).
Platelet-derived growth factors (PDGFs) and PDGF receptors (PDGFRs)

Platelet-derived growth factors (PDGFs), first identified as a constituent of whole blood serum and purified from human platelets, is a family composed of disulfide-linked homo- and heterodimer polypeptide chains (Heldin and Westermark, 1999; Westermark and Wasteson, 1976). The growth factors were originally described as major mitogens for fibroblasts, smooth muscle cells and glia cells in culture (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976).

Four polypeptide chains (PDGF-A, PDGF-B, PDGF-C and PDGF-D), encoded by four different genes, have been discovered to form five different dimeric isoforms; PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB (Andrae et al., 2008; Fredriksson et al., 2004). Whereas human PDGFA and PDGFB genes, carrying seven exons each, are located on chromosomes 7 (Betsholtz et al., 1986) and 22 (Dalla-Favera et al., 1982; Swan et al., 1982) respectively, PDGFC and PDGFD are expressed on chromosomes 4 and 11 (Uutela et al., 2001). For both PDGFA and PDGFB, exon 1 encodes the signal peptide while exons 2 and 3 encode the N-terminal precursor sequence that gets intracellularly processed and removed before secretion (Fredriksson et al., 2004). The mature part of the growth factor core domain is encoded by exons 4 and 5 whereas exon 6 encodes the C-terminal extension (also known as C-terminal retention motif) that is crucial for extracellular matrix (ECM) binding (Figure 3). Structurally, all PDGFs share a highly conserved growth factor core domain essential for peptide dimerization, receptor binding and receptor activation.

The PDGF ligands exert their biological effects on PDGF receptor -α (PDGFRα) and -β (PDGFRβ) (Andrae et al., 2016; Gladh et al., 2016; Hellström et al., 1999; Lindahl et al., 1997b). These two receptors belong to the superfamily of receptor tyrosine kinases and are structurally composed of five extracellular immunoglobulin domains and two divided intracellular tyrosine kinase domains (Andrae et al., 2008) (Figure 4). In their inactive state, the receptors occur as monomers. However, upon ligand binding they undergo dimerization and transphosphorylation occurs at cytoplasmic kinase residues (Kazlauskas and Cooper, 1989; Kelly et al., 1991). This in turn creates docking sites for Src homology 2 (SH2) domain containing molecules to bind to phosphotyrosine residues on the receptor (Claesson-Welsh, 1996), allowing
for further downstream signaling cascade events. The activation of the PDGF-FRs engage several signaling pathways, including PI3K (Kazlauskas and Cooper, 1989), Ras-MAPK (Heldin et al., 1998) and PLCγ (Sultzman et al., 1991). Cellular responses activated by the different signaling pathways involve cell motility and actin cytoskeleton rearrangement (Wennström et al., 1994), cell growth and cell differentiation (Heldin et al., 1998).

The dimerized receptors can appear in one out of the three following constellations: PDGFR-α, PDGFR-β and PDGFR-αβ (Heldin and Westermark, 1999). Whereas PDGFR-α has been demonstrated to interact with PDGF-AA (Lindahl et al., 1997b) and PDGF-CC (Andrae et al., 2016) in vivo, PDGFR-β interacts with PDGF-BB (Hellström et al., 1999) and PDGF-DD (Gladh et al., 2016). PDGFR-αβ is believed to interact with the heterodimer PDGF-AB (Heldin and Westermark, 1999). However, these observations are based on in vitro studies.

![Figure 3](image)

**Figure 3.** Schematic drawing of human PDGF gene, located on chromosome 22. The gene is composed of 7 exons where exons 4 and 5 encodes the growth factor core domain that interacts through dimerization with the receptor. Exon 6 encodes the C-terminal retention motif required for the interaction with extracellular matrix components.

This thesis will from this point onwards focus on the roles of PDGF-B and PDGFRβ under physiological and pathophysiological conditions. The tissue expression of the ligand and receptor vary and the physiological roles of their interactions have mainly been attributed to developmental processes. Mapping of the brain vasculature in adult mice using single cell RNA sequencing (scRNASeq) analysis shows that Pdgfb is mainly expressed by ECs, although lower expression is also seen in neurons (Sasahara et al., 1991), VSMCs and microglia (Vanlandewijck et al., 2018; Zeisel et al., 2018). EC-restricted Pdgfb expression is also found in the lung and other (but not all) organs (Tabula Muris Consortium et al., 2018). The expression of the receptor is confined to pericytes, VSMCs and fibroblasts in the mouse adult brain vasculature (Vanlandewijck et al., 2018) and different cell types (e.g. pericytes, VSMCs,
fibroblasts, stromal cells) in several other organs (Tabula Muris Consortium et al., 2018).

Figure 4. Schematic representation of PDGFR-β protein. The receptor is composed of five extracellular Ig domains, a transmembrane domain and two split intracellular tyrosine kinase domains. Upon ligand binding, the receptor dimerizes and becomes phosphorylated on tyrosine residues, which in turn triggers downstream signaling cascades and diverse biological effects.

The role of PDGF-B and PDGFR-β in blood vessel formation

The development of blood vasculature can be divided in vasculogenesis, the de novo formation of tubular vessels by ECs, and angiogenesis, the formation of new blood vessels through remodeling and sprouting of pre-existing ones. Numerous molecular cues in the form of paracrine, juxtacrine and endocrine ligands play vital roles and are required for cell-cell communications during both vasculogenesis and angiogenesis. The main regulator of vascular development is vascular endothelial growth factor-A (VEGF-A) (Connolly, 1991; Gospodarowicz et al., 1989), a ligand that is produced by neural cells in the CNS and upregulated by hypoxia (Stone et al., 1995).

The recruitment of mural cells, facilitated by PDGF-B/PDGFR-β signaling is also paramount for the development and stability of blood vessels (Armulik et al., 2010; Daneman et al., 2010). PDGF-B, produced and secreted by endothelial tip cells at the leading position of the angiogenic sprout, binds
through the retention motif to heparan sulfate proteoglycans (HSPG) in the ECM and creates a gradient of signaling cue along the developing vasculature (Abramsson et al., 2007; Hellström et al., 1999; Lindahl et al., 1997a; Lindblom et al., 2003) (Figure 5). The ligand exerts its effect through paracrine signaling on PDGFRβ positive VSMCs and pericytes and allows for their proliferation and migration and eventually guides them along the newly formed vessel.

The physiological roles of PDGF-B/PDGFR-β signaling were established in early studies where either the ligand or receptor were ablated in mice (Levéen et al., 1994; Soriano, 1994). Complete loss of PDGF-B or PDGFR-β in mice results in perinatal lethality. The pups die during late gestation due to widespread hemorrhage, general edema and organ failure. Subsequent findings report the failure of mural cell recruitment and the enlargement of blood vessels in these mice, linking PDGF-B/PDGFRβ signaling to blood vessel formation during embryonic development (Hellström et al., 1999; Lindahl et al., 1997a).

Figure 5. The physiological role of PDGF-B/PDGFR-β signaling in blood vessel formation. During angiogenesis, PDGF-B is produced and secreted by endothelial tip cells at the sprouting front. The ligand exerts its effect on PDGFR-β positive mural cells and allows for their proliferation, migration and ultimately for their investment in the vessel wall. The binding of PDGF-B to extracellular matrix components, through the retention motif, is crucial for the formation of a growth factor gradient that acts as signaling cue for mural cells.

Using constitutive knockout (KO) approaches to specifically delete PDGF-B from ECs results in mice that are viable and reach adulthood (Bjarneåård et al., 2004; Enge, 2002). However, these mutants have impaired pericyte recruitment to brain capillaries, with 50-90% pericyte loss (Enge, 2002). In addition, they exhibit kidney, placental and cardiac abnormalities, confirming the importance of PDGF-B signaling during embryonic development (Bjarneåård et al., 2004; Enge, 2002). Additionally, postnatal deletion of EC-
derived PDGF-B using tamoxifen inducible Cre-loxP system lead to pericyte drop-out on retinal capillaries and increased blood-retinal barrier permeability (Park et al., 2017; Pitulescu et al., 2010). Interestingly, neuron-specific ablation of PDGF-B result in viable mice which display no apparent effect on behavior, pericyte loss or other vascular abnormalities (Enge et al., 2003).

The Pdgfb retention motif KO (Pdgfbret/ret) mouse model, where a stop codon in exon 6 of the Pdgfb gene results in the deletion of the retention motif, has been extensively used to study the postnatal effects of impaired PDGF-B signaling (Lindblom et al., 2003). The mutation renders a biologically active PDGF-B protein that due to its inability to bind HSPG fails in proper recruitment of pericytes to the developing brain capillaries. The Pdgfbret/ret mice are adult viable and present two hallmarks of reduced brain pericyte coverage (25% as compared to control): capillary diameter enlargement and increased BBB permeability (Armulik et al., 2010).

Pathological roles of PDGF-B and PDGFR-β

Although the pathological roles of PDGF-B and PDGFR-β are still poorly understood, mutations in their respective genes have been linked to some rare tumors and leukemias (Demoulin and Essaghir, 2014; Östman and Heldin, 2007). The tumors associated with PDGFβ and PDGFRβ are of mesenchymal and glial origin (sarcomas and gliomas, respectively), reflecting the expression patterns of the ligand and receptor.

Chromosomal translocation affecting the PDGFB gene is seen in a rare form of skin tumor of intermediate malignancy, dermatofibrosarcoma protuberans (DFSP). In DFSP the translocation affects exon 2 of the PDGFB gene on chromosome 22, which fuses with the high expressing collagen 1 alpha 1 (COL1A1) gene on chromosome 17 (SIMMON, 1997). The fused gene encodes a mature and functional PDGF-B protein that activates PDGFR-β through paracrine and autocrine signaling (Shimizu et al., 1999). Treatment with the tyrosine kinase inhibitor, imatinib, has shown clinical effects in patients diagnosed with DFSP (McArthur et al., 2005).

Infantile myofibromatosis (IM) is a proliferative fibrous tumor affecting infants and children and found in soft tissue that has been linked to missense mutations in genes including PDGFRB and NOTCH3 (Cheung et al., 2013; J. Lee, 2013; Martignetti et al., 2013). The causative PDGFRB mutations renders a receptor that is constitutively active in the absence of ligand and sensitive to tyrosine kinase inhibitors such as imatinib and nilotinib (Arts et al., 2016).

Interestingly, loss-of-function (LoF) mutations in both PDGFB and PDGFRB were simultaneously discovered to be causative for primary familial brain calcification (PFBC) (Keller et al., 2013; Nicolas et al., 2013b).
Primary familial brain calcifications (PFBC)

Intracranial calcifications
The findings of intracranial ectopic calcifications unaccompanied by any clinical manifestations are common in patients undergoing computed tomography scan (CT) (Kiroglu et. al., 2010). Incidents of non-pathological calcifications are discovered during routine CT scans in approximately 20% of the population and different brain regions are affected to different extent (Yamada et al., 2013). In the vast majority of cases, the calcifications develop in an age-dependent manner and are detected in adults or elderly patients.

Rare congenital conditions associated with brain calcifications occur in diseases like Sturge-Weber syndrome (SWS) (Maton et al., 2010) and tuberous sclerosis (TS) (Thibaut et al., 1993). SWS results from cerebral vascular malformations in the cerebral cortex causing venous hypertension and subsequent hyperfusion of the underlying tissue and chronic cerebral ischemia. TS is an autosomal dominant disease characterized by epilepsy and mental retardation where calcified lesions are found in the white matter (Kiroglu et. al., 2010).

In 1850, Delacour first documented the findings on vascular calcifications in the basal ganglia after pathological examination of a deceased patient that had shown clinical symptoms of tremors and weaknesses in lower extremities (Delacour, 1850). Later, Bamberger and Fahr also reported discoveries of bilateral calcifications in different brain regions of patients whom had been presented with neurological symptoms (Manyam, 2005). Following his reports, Fahr’s name became associated with bilateral brain calcifications. In fact, one of the many names that has been used for the condition is Fahr’s syndrome. In recent years, the linking of mutations in specific genes to the disease, has been reflective in the terminology and nowadays the preferred nomenclature is PFBC. Prior to that, it was often referred to idiopathic basal ganglia calcifications.

Pathophysiology of PFBC
PFBC, a rare and age-dependent neurodegenerative condition, is manifested by the formation of bilateral calcium deposits primarily in the basal ganglia, thalamus brainstem and cerebellum (Manyam et al., 2001; Norman and
Although the prevalence of the disease remains mostly unknown, some reports have estimated it to be <1/1,000,000 in the population (Nicolas et al., 2018). The ambiguity to ascertain a precise number may stem from the fact that relatively few cases have been reported.

The deposits are composed of calcium (hydroxyapatite) but traces of silver, aluminum, arsenic, copper, cobalt, magnesium, silver, molybdenum, iron, lead, phosphorus, manganese and zinc has also been reported to be present (Duckett et al., 1977; Smeyers-Verbeke et al., 1975). Levels of calcium, phosphorous and parathyroid hormones are normal and the systemic mineral metabolism is unaltered (Betsholtz and Keller, 2014; Samuels et al., 2018).

Bilateral calcifications in PFBC form along capillaries, arterioles and venules along with associated gliosis from reactive astrocytes and microglia (Kozik and Kulczycki, 1978; Miklossy et al., 2005). Moreover, electron microscopy (EM) analysis revealed the presence of mineral deposits within perivascular cells surrounding the blood vessels in samples from patients (Kobayashi et al., 1987). Although signs of neuroinflammation and extravasation of plasma proteins have been reported, the general physiological state of neurons seems to be conserved (Miklossy et al., 2005). Other pathological findings in PFBC patients include vasogenic edema, dilation of lateral ventricles, reduction in glucose metabolism and regional blood flow changes (Gomez et al., 2016; Kozik and Kulczycki, 1978; Shouyama et al., 2005).

It is, however, import to stress that many of these case reports were published before the genetic component of the disease was known. The heterogeneous pathologies of the reports associated with brain calcifications may as well have represented different disease etiologies.

Diagnosis and clinical features

Diagnosis of PFBC is based on neuroimaging techniques, signs of progressive neurological dysfunction, absence of infectious or toxic agents in CSF and normal levels of minerals (e.g. calcium, magnesium, phosphorous), serum parathyroid hormone and heavy metal concentrations in urine and blood (Bonazza et al., 2011). Visualization of calcifications using brain CT scans appear more progressive in older individuals and calcified areas give a low signal intensity on magnetic resonance imaging (MRI) (Avrahami et al., 1994). While CT scans provide more sensitive detection of calcifications (Yamada et al., 2013), MRI offers a more detailed anatomical imaging (Avrahami et al., 1994). To distinguish PFBC-related calcifications from other causes of calcifications using imaging techniques is generally not possible. However, the localization and appearance may be useful guidelines in the diagnostics (Livingston et al., 2013). After the discoveries of the genetic contributions to the disease, genomic tests are performed to determine the diagnose.
The clinical manifestations of PFBC, albeit presented in a heterogenous manner, are restricted to the nervous system. Findings of bilateral calcifications in asymptomatic individuals have been reported in ~30% of the cases (Huang et al., 2018; Manyam et al., 2001; Samuels et al., 2018). The relatively high frequency of asymptomatic cases may be a contributing factor to the underreporting nature of PFBC. Although most of the incidences have been reported in adults, the clinical onset ranges from early childhood to ~80 years of age (Nicolas et al., 2015) and the male:female ratio is estimated to be 2:1 (Manyam, 2005). The most frequent clinical symptoms accompanying the condition are neuropsychiatric symptoms (including behavioral disturbances, psychosis and cognitive impairment) (Adam et al., 1993; Chiriaco et al., 2018) and movement disorders (such as parkinsonism and tremor) (Rohani et al., 2017). Other less frequent signs are speech impairments, seizures, headache and vertigo (Guo et al., 2019; Y. J. Lee et al., 2018). It has been proposed that symptomatic patients are presented with higher calcification scores compared to asymptomatic patients (Nicolas et al., 2013a) and the correlation of specific phenotypes to the different genetic mutations have been observed (Batla et al., 2017).

The genetic mutations causing PFBC

Although sporadic cases have been reported for the condition, a growing number of studies have associated PFBC to pathogenic mutations in five different genes (below listed). Whereas mutations in four of the causative genes (\textit{SLC20A2}, \textit{PDGFB}, \textit{PDGFRB} and \textit{XPR1}) are inherited in an autosomal dominant manner, the latest one (\textit{MYORG}) follows an autosomal recessive fashion (Figure 6).

- **Solute carrier family 20 member 2 (\textit{SLC20A2})**: mutations in \textit{SLC20A2} were the first ones to be described as causative for the disease (Wang et al., 2012). Seven LoF mutations (five missense, one frameshift and one deletion) were discovered in seven families of different ancestry. Since that first discovery, more than 60 different variants have been reported in the literature and \textit{SLC20A2} mutations account for the most frequent ones in PFBC incidences (Oliva et al., 2019). The gene is located on chromosome 8 and encodes the type III sodium-dependent inorganic phosphate (Pi) transporter 2 (PiT2), a transmembrane protein involved in phosphate homeostasis in various cell types and tissues including the brain (Kavanaugh and Kabat, 1996; Lagrue et al., 2010). A dysfunctional PiT2 protein may lead to inadequate uptake of Pi by cells, consequently leading to buildup of calcium phosphates in the tissue and subsequent formation of calcifications (Wang et al., 2012).
• **PDGFRβ**: one missense mutation in the *PDGFRβ* gene was discovered in two related French patients with a family history of PFBC (Nicolas et al., 2013b). Additional screening in 19 patients revealed one more missense mutation in a sporadic case. Later, two more missense mutations in patients without a family history of PFBC were discovered to be pathogenic (Nicolas et al., 2013a; Sanchez-Contreras et al., 2014). All mutations have been predicted to be LoF and disease-causing due to conserved amino acid substitutions leading to reduced autophosphorylation and receptor levels (Sanchez-Contreras et al., 2014).

• **PDGFB**: two pathogenic mutations, one nonsense and one missense, were detected by genome sequencing in two different families (Keller et al., 2013). Sanger sequencing revealed the presence of four more additional variants in families of mainly European ancestry. Two more sporadic cases with new variants were discovered in French patients, one being a de-novo mutation (Nicolas et al., 2014a) and the other being a rearrangement, deleting exons 3-6 of the *PDGFB* gene (Nicolas et al., 2014b).

• **Xenotrpic and polytropic retrovirus receptor 1 (XPR1)**: out of six detected mutations, four deleterious missense variants in the *XPR1* gene, were detected in five PFBC patients from unrelated families (Legati et al., 2015). One more variant was identified in a patient not long after (Anheim et al., 2016). The gene encodes a multi-pass transmembrane protein involved in P_1 export and is conserved among most species (Kavanaugh et al., 1994). The mutations were identified to be located in a regulatory domain of the protein, leading to insufficient cellular phosphate export (Legati et al., 2015). Gene expression analysis of *XPR1* revealed a wide distribution in the mouse brain and functional analysis indicated a potential interaction of the protein with PDGFR-ß (X.-P. Yao et al., 2017).

• **MYORG**: the most recent identification of gene-specific mutations associated with PFBC was found in *MYORG* (X.-P. Yao et al., 2018). Unlike the mutations in the previously described genes, mutations in *MYORG* are inherited in an autosomal recessive manner in patients. Nine variants were found and among those four were classified as missense mutations in highly conserved regions, three nonsense mutations and two were rearrangement mutations. The gene encodes a lamina-associated transmembrane protein, located in the nuclear envelope, that belongs to the glycosidase family 31 (Datta et al., 2009). Specific expression was found in astrocytes in the brain and inactivation of the enzyme was predicted to
lead to insufficient protein glycosylation and metabolism, ultimately trig-
gerating the formation of calcifications (X.-P. Yao et al., 2018).

Figure 6. Physiological roles of the PFBC-causative genes in humans. While
SLC20A2, XPR1, PDGFB and PDGFB and PDGFRB all have shown to be inherited
in an autosomal dominant fashion, MYORG is inherited in an autosomal recessive
manner. The products of the five genes are structurally and functionally diverse and
the mechanism by how pathogenic mutations in them causes PFBC is currently not
known.

Mouse models of PFBC
A few mouse models of PFBC, mimicking the human condition, have been
established over the years in order to increase the understanding of the disease
etiology. Slc20a2 homozygous KO mice were demonstrated to develop bilat-
eral brain calcifications in agreement with reported findings in humans (Jen-
sen et al., 2013). The authors used histological approaches to identify mineral
nodules associated with blood vessels and macrophage/microglial reactivity,
mainly in the thalamus along with a few findings in the cortex and basal gan-
glia. Subsequent analysis of the Slc20a2-/- mice revealed the presence of ele-
vated levels of inorganic phosphates in the CSF (Jensen et al., 2016; Walling-
ford et al., 2016) and placental calcifications accompanied by abnormal pla-
cental vasculature and increased BM deposition. Wallingford et. al. also re-
ported the findings of nodules in adult heterozygous Slc20a2+/-.mice, formation
of ocular calcifications, intact BBB integrity, high expression of Slc20a2 in VSMCs and the formation of calcifications in arteriolar VSMCs in the lymphatic-pathway (Wallingford et al., 2016). In sharp contrast, a more recent study failed in detecting nodules in heterozygous mice (Jensen et al., 2018). Moreover, the study suggests that the calcifications had an intracellular origin in pericytes and astrocytes and reported an increase in endogenous IgG around the calcifications, signs of dysfunctional BBB properties.

Figure 7. Pdgfb<sup>ret/ret</sup> as a mouse model for PFBC. The deletion of the C-terminal retention motif renders a biologically active Pdgf-b protein. However, the ligand fails in creating a proper growth factor gradient resulting in impaired recruitment of mural cells to the developing vasculature. The Pdgfb<sup>ret/ret</sup> mice are adult viable and develop vessel associated calcifications in deep brain regions with age, mimicking human PFBC condition. They also display vessel enlargement and dysfunctional BBB properties.

Detailed analysis of the hypomorphic Pdgfb<sup>ret/ret</sup> mice revealed the formation of age-dependent, calcifications found in the dorsal pons, thalamus, hypothalamus and midbrain (referred in paper I as calcification-prone regions), similar in anatomical locations and appearance as those described in human PFBC patients (Keller et al., 2013) (Figure 7). The Pdgfb<sup>ret/ret</sup> mice are presented with severe pericyte loss (Lindblom et al., 2003), dilated blood vessels and an increase in BBB permeability (Armulik et al., 2010). In addition, the mice exhibit severe retinal defects including disorganized vasculature, fibrosis and degeneration of nuclear and photoreceptor layers (Lindblom et al., 2003). Moreover, glomerular defects with decreased mesangial cell recruitment were observed postnatally but later reversed and restored to normal levels. The Pdgfb<sup>ret/ret</sup> mice also display VSMC deficiency however VSMC loss is less pronounced than pericyte loss (Lindblom et al., 2003) and defects in astrocyte end feet polarization (Armulik et al., 2010). Calcifications in Pdgfb<sup>ret/ret</sup> mice are surrounded by cells expressing osteoblast and osteoclast markers, resulting in a neurotoxic astrocyte response (Zarb et al., 2019). Furthermore, the
nodules are restricted to the brain and the mice display some behavioral alterations also reported in a subset of human PFBC cases.

In conjunction with the identification of recessive mutations in the MY-ORG gene causing PFBC in humans, it was also discovered that brain calcifications develop in 9 month old Myorg KO mice in the thalamus region (X.-P. Yao et al., 2018). Taking into consideration that these mouse models represent mutations in genes that are structurally and functionally diverse, it is currently unclear to what extent they present the same disease etiology. Nevertheless, they are important tools in taking us further in understanding PFBC.
Present investigations

Paper I

Functional characterization of germline mutations in PDGFB and PDGFRβ in primary familial brain calcification

The discovery that mutations in PDGFB and PDGFRβ are causative for PFBC has assigned new pathogenic roles for the ligand and receptor. Therefore, we aimed to functionally analyze several of the known PFBC-related PDGFB and PDGFRβ mutations in vitro in paper I. Our investigation showed that all six analyzed PDGFB mutations lead to complete loss of PDGFB function, either through abolished protein synthesis or through defective binding and/or stimulation of PDGFRβ.

By functionally assessing three PDGFRB mutations, we found that they had more diverse consequences, ranging from almost totally abolished autophosphorylation to reductions in protein levels and specific changes in PLCγ activation.

We also explored the outcomes of reduced Pdgfb or Pdgfrb transcript and protein levels in Pdgfb<sup>−/−</sup>, Pdgfrb<sup>−/−</sup> and Pdgfb<sup>−/+</sup>;Pdgfrb<sup>−/−</sup> mice on the formation of calcifications. Calcified nodules were neither detected in any of the abovementioned genetic models, nor did they develop in Pdgfrb<sup>redye/redye</sup> mice, which show a 90% reduction of PDGFRβ protein levels.

Analysis of pericyte coverage in calcification-prone and non-calcification-prone brain regions in Pdgfb<sup>ret/ret</sup> mice revealed that calcification-prone brain regions in Pdgfb<sup>ret/ret</sup> mice had a higher pericyte coverage and a more intact BBB compared to non-calcification-prone brain regions. This suggests that region-specific susceptibility factors may reside in PFBC pathogenesis that are distinct from pericyte coverage and BBB deficiency.
Paper II

Astrocyte – microglial association and matrix deposition define common pathogenic events in primary familial brain calcification

While the genes causing PFBC have diverse physiological functions, the development of brain calcifications have been reported for Slc20a2, Pdgfb and Myorg mutants, recapitulating the human condition. The aim of paper II was to perform an in-depth analysis of the calcifications formed in two of the abovementioned models, namely the Pdgfb<sup>ret/ret</sup> and Slc20a2<sup>-/-</sup> mice.

By use of transmission electron microscopy and immunofluorescence we could show that the nodules display a multi-layered ultrastructure and that they are in direct contact with reactive astrocytes and microglia in both models.

Through proteomic and immunofluorescence analysis, we found a common molecular composition of the nodules, with the presence of proteins implicated in bone homeostasis. We also found that the nodules were composed of proteins that previously have not been linked to tissue mineralization. In addition, with immunofluorescence analysis, we could confirm the close association of astrocytes and microglia to the calcifications.

We also detected clear differences between the two models. While Pdgfb<sup>ret/ret</sup> mice developed large, solitary nodules, the deposits in Slc20a2<sup>-/-</sup> mice were multi-lobulated and occurred in clusters. Moreover, the nodular distribution in Pdgfb<sup>ret/ret</sup> mice were concentrated to the dorsal pons, thalamus, hypothalamus and midbrain. In contrast, they were more spread out in deeper brain regions in Slc20a2<sup>-/-</sup> mice and in few cases even detected in the cerebral cortex. This observation might reflect local anatomical differences leading to differences in mineral deposition susceptibility.

Assessment of pericyte coverage and blood-brain barrier integrity in Slc20a2<sup>-/-</sup> revealed both to be intact, suggesting that these two features, both compromised in Pdgfb<sup>ret/ret</sup> mice, are likely not the causal triggers of PFBC pathogenesis. Instead, we propose that gene expression and spatial correlations point to astrocytes as culprit cells in PFBC.
Paper III

Endothelium-derived PDGF-B is essential for adult brain pericyte and vascular smooth muscle cell maintenance

The role of PDGF-B has mainly been attributed to mural cell recruitment and vessel maturation during developmental stages in the brain. However, what role endothelial sources of PDGF-B has on mural cell stability and maintenance in adult, quiescent vasculature is still not known.

In paper III, we sought to investigate the role of endothelium restricted PDGFB on mural cell maintenance in fully developed and quiescent adult brain vasculature. In addition, we studied whether the adult loss of Pdgfb is sufficient to cause increased BBB permeability and development of brain calcifications in mice.

For that, we took a tamoxifen-inducible approach to delete Pdgfb specifically in endothelial cells by crossing either Pdgfb\textsuperscript{floxed/floxed} or Pdgfb\textsuperscript{floxed/-} mice with Cdh5-CreERT2 mice (Pitulescu et al., 2010). This breeding strategy gave rise to litters with Cre negative offspring analyzed as controls (Ctrl) and Cre positive mice with either two floxed alleles or one floxed and one null allele analyzed as endothelial cell PDGF-B knock-outs (Pdgfb\textsuperscript{ECKO}). We induced 2-month-old mice and sacrificed at 4 months for early time-point analysis and 12- and 18-months for late time-point analysis.

In paper I we showed that 50% loss of Pdgfb gene is not sufficient to significantly alter pericyte numbers or lead to the formation of calcifications in mice. In order to confirm sufficient gene deletion efficiency, we performed qPCR on isolated microvascular fragments from one brain hemisphere. The other hemisphere was utilized for either permeability studies or mural cell coverage studies by immunofluorescence. We also screened several late time-point litters for the appearance of calcification by staining with two of the calcification markers we found in paper II.

In this study, we demonstrate that endothelium-derived PDGFB is crucial for mural cell maintenance also in adult mouse brain. Deletion of endothelial Pdgfb (>80% downregulation in early time point and >95% in late time point) resulted in nearly 50% pericyte loss, as evident by pericyte coverage quantifications and decreased expression levels of pericyte/mural cell genes. Although VSMC morphology was altered, they seemed affected to a lesser degree than the pericytes, an observation also made in constitutive Pdgfb and Pdgfrb mutants (Hellström et al., 1999). We did not detect any signs of vessel enlargement, BBB disruption (assessed by intravenously injected Alexa Flour 555 cadaverine accumulation and Fibrinogen staining) or formation of calcifications.
A dysfunctional brain vasculature in several cerebrovascular disorders is a major cause of death in many parts of the world. However, in order to understand how the vasculature changes under disease conditions, it is fundamental to define the molecular signature of brain vascular cells in a normal physiological state.

In paper IV, we used single-cell transcriptomics to provide molecular definitions for the principal types of blood vascular and vessel-associated cells in the adult mouse brain. For this, we took advantage of the following reporter mice to capture major vascular and vessel-associated cell types from the adult brain: Cldn5-GFP, Pdgfrb-GFP; Cspg4-DsRed, Tagln-Cre; R26-stop-tdTomato and Pdgfra-H2BGFP. We sorted single cells in 384-well plates and created sequencing libraries with Smart-seq2 chemistry. After sequencing and alignment, the data was clustered using the BackSPIN algorithm and transformed in an online searchable database (http://betsholtzlab.org/VascularSingleCells/database.html).

Through this investigation, we found a gradual phenotypic change (zonation) along the arteriovenous axis. While the endothelial cells present a transcriptomic continuum along the anatomical axis, mural cells displayed a punctuated expression profile along the axis, with an abrupt transition from VSMC to pericyte. We also establish that pericytes possess a single identity in the healthy adult mouse brain and that they are organotypic by comparing the vasculature between brain and lung. Noticeably, brain pericytes abundantly express SLC, ABC and ATP transporters while the same markers are absent or low expressed in lung pericytes. This shows that pericytes play an integral part in BBB transport. Moreover, we define a population of perivascular fibroblast-like cells that are present on all vessel types except capillaries.
Future perspectives

Our understanding of how the vasculature and vessel associated cells function in health and disease is significantly improving. Nevertheless, it is evident that with increasing knowledge, further questions arise that need to be addressed. The work presented herein has mainly focused on exploring the specific circumstances resulting in the formation of vessel associated pathological brain calcifications in mouse models of PFBC.

With the aid of techniques such as scRNAseq, it is now possible to profile individual cells on a molecular level. This paves the way for future investigations to unravel what happens to those same cell types under pathological conditions that might explain disease mechanisms. As a continuation of the study performed in paper IV, the lab is currently undertaking a similar approach to transcriptomically define endothelial cells and mural cells in \( Pdgfb^{ret/ret} \) mice. Interestingly, in paper I we discovered that pericyte drop-out and BBB impairment is more pronounced in non-calcification prone regions compared to calcification-prone regions. This particular finding raises the question to whether the mural cells and underlying ECs associated with nodules are dysfunctional to certain degrees in \( Pdgfb^{ret/ret} \) mice, and scRNAseq has the potential to provide us an insight.

In contrast, pericyte coverage and BBB functions were found to be intact in \( Slc20a^{-/-} \) mice and this might suggest that these two factors are less significant for the formation of calcifications. A more probable scenario is that astrocytes and microglia play crucial roles in the calcification forming process. The two cell types were consistently observed to oppose the calcified nodules in paper II. Moreover, given that astrocytes express four out of the five PFBC-causing genes (to varying degrees) is intriguing enough to assign them a central role. These particular observations encouraged us to follow up on paper II by assessing the expression profiles of astrocytes and microglia in \( Pdgfb^{ret/ret} \) mice using scRNAseq. We have isolated single cells from both calcification prone and non-calcification prone regions and sorted them in 384-well plates from the following transgenic lines: astrocytes from \( Pdgfb^{ret/ret} \), Aldh1l1-ttdTomato and microglia from \( Pdgfb^{ret/ret} \), Cx3cr1-GFP. These experiments may provide insight into whether astrocytes and microglia in a brain calcification model express genes that are otherwise associated with osteoblasts and osteoclasts.

The findings in paper III suggests that loss of PDGF-B proteins is required during developmental stages in order to have a negative impact on BBB
integrity and for the formation of calcifications. However, we found that pericyte coverage and mural cell phenotype were significantly altered in adult induced $Pdgfb^{ECKO}$ mice. Although the underlying vasculature seems normal in size, shape and density in $Pdgfb^{ECKO}$, it would be of great interest to explore whether pericyte loss in this model affects other vascular properties such as blood flow, vascular tone and increased immune cell infiltration.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)