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2019

*Document Version:*
Publisher's PDF, also known as Version of record

Link to publication

*Citation for published version (APA):*
Roth, M. (2019). *Functions of Pericytes in Ischemic Stroke*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Lund]. Lund University: Faculty of Medicine.

*Total number of authors:*
1

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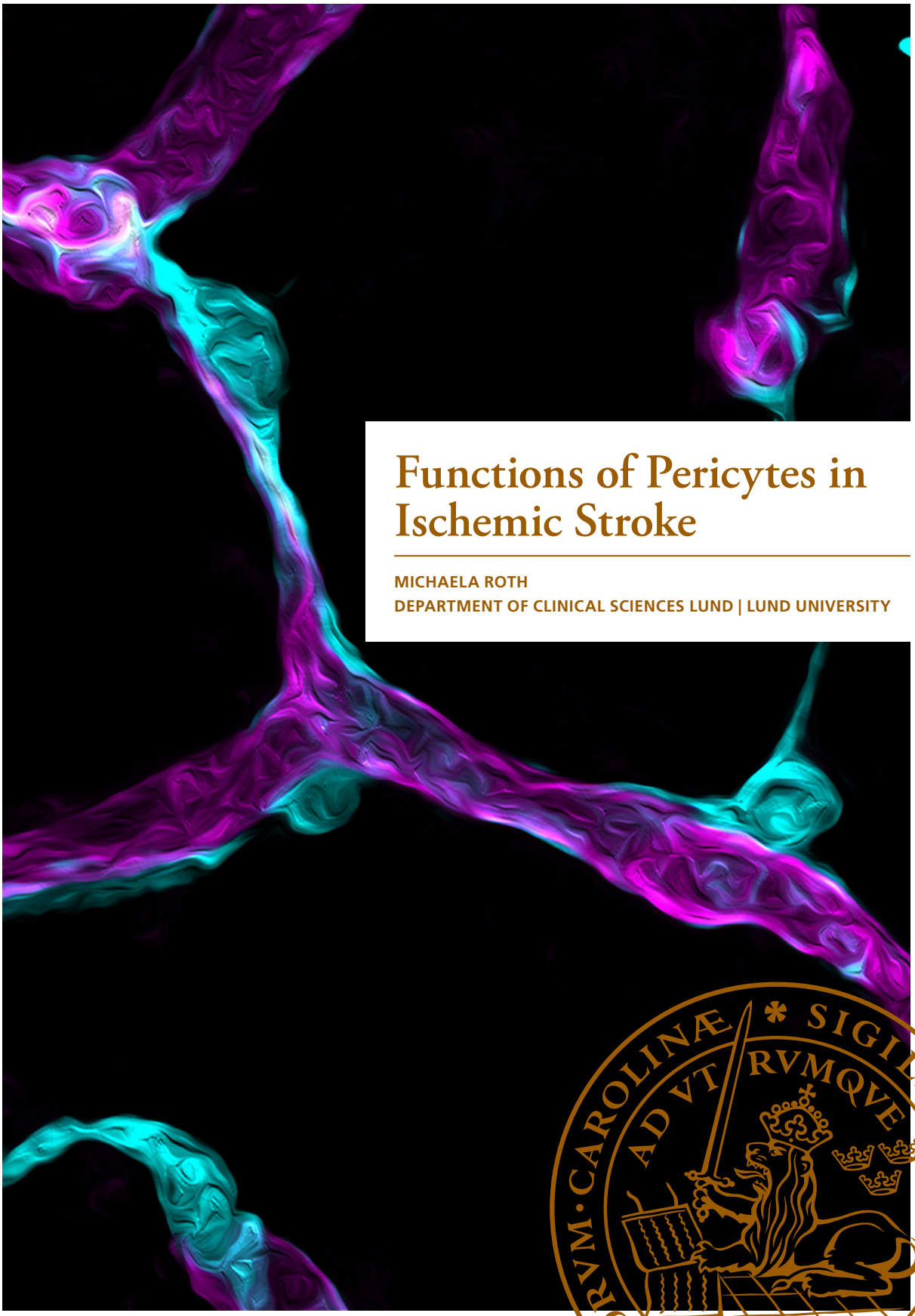

# Functions of Pericytes in Ischemic Stroke

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# Functions of Pericytes in Ischemic Stroke

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Michaela Roth


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DOCTORAL DISSERTATION

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To be defended on 25th October 2019, at 9:00, Segerfalksalen, Wallenberg Neuroscience Center, Lund University, Lund, Sweden.

*Faculty opponent*
**Professor Martin Lauritzen**
Department of Neuroscience
University of Copenhagen, Denmark

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|---|---|
| **Organization**<br>LUND UNIVERSITY<br>Faculty of Medicine<br>Department of Clinical Sciences<br>Translational Neurology Group | **Document name**<br>Doctoral Thesis |
| | **Date of issue** October 25, 2019 |
| **Author**<br>Michaela Roth | **Sponsoring organization** Lund University |


**Title:** Functions of Pericytes in Ischemic Stroke


**Abstract**

Ischemic stroke remains one of the leading causes of death and disability worldwide, and its burden is predicted to further increase due to the aging population. The only available treatments, thrombolysis or thrombectomy, can only be applied within a limited time window after stroke onset, and thus are applicable only to a small proportion of stroke patients. Therefore, there is an increasing need for new therapeutic approaches. In addition to neuronal cell death, the stroke pathology is characterized by the breakdown of the blood-brain barrier (BBB), resulting in the accumulation of blood-derived components within the brain, further aggravating neuronal cell death. Several repair mechanisms occur within the brain after the ischemic insult, including vascular remodeling to reestablish the blood flow as well as scar formation to both replace the injured tissue and contain the inflammation within the injured ischemic core.

Pericytes, perivascular cells lining capillaries, have increasingly gained interest as a novel target cell type. This is due to their multiple functions after stroke that include maintenance of the BBB and their participation in vascular remodeling and scar formation. Pericytes undergo several morphological and phenotypic changes in stroke. One of these changes is the expression of Regulator of G-protein signaling 5 (RGS5), a protein that is upregulated in pericytes after stroke before they detach from the vessels, suggesting its involvement in this detachment process. However, the time course of the pericyte response in relation to other vascular changes, and the impact that loss of RGS5 has on pericytes and their function during the different stages of stroke are not yet known.

Using a permanent stroke model in mice, we established the temporal sequence of the pericyte response in relation to other vascular events after ischemic stroke. Pericytes were the first vascular cells to respond to ischemic stroke by either undergoing cell death or activation. Most importantly, the pericyte response preceded loss of tight junction (TJ) proteins, endothelial cell death and BBB leakage. Loss of RGS5 in pericytes resulted in increased pericyte numbers and coverage. In the acute phase, the increased pericyte coverage in RGS5-knock out (KO) mice prevented TJ loss and reduced the BBB breakdown. This was associated with a reduction in neuronal cell death after stroke. In the chronic phase, loss of RGS5 reduced detachment of platelet-derived growth factor receptor ß (PDGFRß)$^+$ pericytes from the vascular wall, resulting in a shift from a parenchymal to a perivascular location of PDGFRß$^+$ cells. This was accompanied by maintenance of PDGFRß-signaling at baseline levels and vessel stabilization as seen by increased vascular density and reduced vascular leakage. Pericytes that migrate into the parenchyma following stroke have been suggested to be involved in scar formation after stroke. However, a reduction in parenchymal PDGFRß$^+$ cells by 50% in RGS5-KO mice did not lead to alterations in the deposition of the extracellular matrix proteins type I collagen and fibronectin; however, it resulted in an earlier maturation of the glial scar.

In conclusion, the results in this thesis identify pericytes as an early responder after stroke. Our studies highlight RGS5 as an important modulator of neurovascular protection in the acute phase and vascular remodeling in the chronic phase after stroke. Targeting pericytes, for example via RGS5, constitutes a potential novel target for therapeutic interventions.



**Key words:** Stroke, pericytes, RGS5, blood-brain barrier, vascular remodeling, scar formation

Classification system and/or index terms (if any)

| | | |
|---|---|---|
| **Supplementary bibliographical information:**<br>Lund University, Faculty of Medicine Doctoral Dissertation Series 2019:99 | | **Language:** English |
| **ISSN** and key title: 1652-8220 | | **ISBN:** 978-91-7619-828-5 |
| Recipient's notes | **Number of pages** 86 | Price |
| | Security classification | |



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# Functions of Pericytes in Ischemic Stroke

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2019

Cover art by Michaela Roth.
Front: Brain pericytes around blood vessels.
Back cover: Watercolor painting representing the vascular tree and the variety of pericytes, at the border between the outside world and the brain

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Faculty of Medicine
Department of Clinical Science Lund

ISBN 978-91-7619-828-5
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2019:99

Printed in Sweden by Media-Tryck, Lund University
Lund 2019


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MADE IN SWEDEN

*To my loved ones,*
*who always believed in me*

*"I believe in you." Words that water flowers.*
- Michael Faudet

*Learn from yesterday, live for today, hope for tomorrow.*
*The important thing is not to stop questioning.*
- Albert Einstein

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*Neurobiology of Disease* (2016); 94:95-105.

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*Plos ONE* (2018); 13(3): e0194146

# Populärvetenskaplig sammanfattning

Stroke kan drabba vem som helst, när som helst. Från en sekund till nästa förändras livet radikalt både för den strokedrabbade och anhöriga.

En stroke, vilket också kan kallas för ett slaganfall, är vanligtvis orsakad av en blodpropp i hjärnan. Denna blodpropp stoppar tillflödet av blod till någon del av hjärnan. Beroende på varaktighet och vilken del av hjärnan som drabbas av en stroke kan patienterna komma att lida av partiell förlamning, problem med språket, eller i värsta fall dö. En viss del av patienterna återhämtar sig fullständigt, men majoriteten måste leva med livslånga åkommor. Därför är det inte överraskande att stroke är en vanlig dödsorsak, men också en av de vanligaste orsakerna till funktionshinder. Den enda behandling som finns idag, innefattar att lösa upp blodproppen så att blodflödet kommer igång igen. Emellertid kan bara 10% av patienterna få denna typ av terapi, därför forskas det intensivt för att hitta nya terapeutiska alternativ.

För att utveckla nya terapier är det viktigt att förstå alla processer som sätts igång efter en stroke. En stroke leder till minskad syretillförsel i den påverkade delen av hjärnan och på grund av detta dör hjärncellerna, de så kallade neuronerna. Dessutom blir blod-hjärnbarriären mer genomtränglig. Blod-hjärnbarriären kontrollerar vanligtvis vilka ämnen som kan komma från blodet in till hjärnan och vice-versa, samt att inga toxiska ämnen kommer i kontakt med de känsliga neuronerna. En av de celltyper som formar blod-hjärnbarriären är de så kallade pericyter, som kramar sig runt blodkärlen och har många vitala funktioner i kroppen. Utöver deras uppgift i blod-hjärnbarriären är pericyter viktig för bildning av nya blodkärl och ärrvävnad efter en stroke.

Syftet med denna avhandling var att undersöka hur pericyter reagerar efter stroke, och om man kan förändra pericyternas svar för att förhindra skador eller för att förbättra läkningsprocessen.

Det visade sig att pericyter är en av de första celltyperna som reagerar efter stroke. Ett av de viktigaste fynden var att pericyters svar på stroke följs av en ökad genomsläpplighet av blod-hjärnbarriären. Vår hypotes var därför att pericyter är en ideal målcelltyp för at förhindra blod-hjärnbarriärens kollaps. I ett annat steg insåg vi att bortagning av en gen i hjärnans pericyter, *rgs5,* modulerar pericyternas svar vid stroke. Mössen som inte producerar RGS5-proteinet, hade ett högre antal pericyter runt blodkärlen. Detta stärker blod-hjärnbarriären, vilket leder till mindre hjärnskador och celldöd efter stroke. Möss utan RGS5 hade också både fler och stabilare blodkärl efter stroke. Intressant nog bidrar pericyter bara minimalt till nybildning av ärrvävnad i hjärnan efter stroke.

I denna avhandling har vi gjort studier på pericyter vid stroke i möss. Normala pericyter är nödvändiga för att skydda hjärnvävnaden. Vid stroke förändras deras funktion och RGS5 spelar en viktig roll i denna process. Pericyterna som saknar RGS5 kan signifikant bättre bidra att skydda blodkärl och nervceller vid stroke.

# Popular Science Summary

A stroke can happen at any time and to anybody. It can also change the life of a person, and their families, from one second to the next.

Stroke is a “brain attack” that, in most cases, is caused by a blood clot that blocks the blood flow to parts of the brain leading to cell death. Depending on the duration and location of the stroke, patients might lose their capacity to speak and move or even die. Some patients recover from a stroke, but the majority remains with some disability, making stroke one of the leading causes of disability worldwide. Today, the only possible treatment is the immediate removal of the blood clot to restore the blood flow and prevent cell/tissue death. However, only a small proportion of patients can receive this treatment; therefore, there is an increasing demand for novel therapeutic approaches.

To develop new therapies, it is crucial to understand all of the processes that occur after a stroke. A stroke leads to reduced oxygen levels in the affected brain areas, and because of this, nerve cells, so-called neurons, die. Apart from neuronal cell death, one of the critical features of stroke is the breakdown of the blood-brain barrier (BBB). This barrier usually controls which substances enter from the bloodstream into the brain, and with this ensures that the sensitive neurons are not exposed to toxic substances. One of the cell types that are part of the BBB are so-called pericytes. Pericytes are cells that wrap around blood vessels and they have many important functions within the brain.

Besides their role in protecting the BBB, pericytes are involved in building new blood vessels, and they have been suggested to contribute to scar formation, both are events that occur after stroke. In this thesis, we are interested in understanding how exactly pericytes react after stroke, and whether we can specifically modify pericytes to either prevent damage or facilitate the recovery process.

We determined that pericytes are one of the first cell types to respond after a stroke. Importantly, pericytes reacted before we saw the effects of the stroke on the vasculature or the breakdown of the BBB. These findings suggested that pericytes could be a promising target to either prevent or reduce the injury after stroke. We found that the removal of a particular gene called *rgs5* is a way to modulate the pericyte response. Mice lacking RGS5 in their brain pericytes had higher numbers of pericytes that remained around blood vessels. This resulted in reduced breakdown of the BBB and hence, less damage after stroke. These mice also had a higher number of blood vessels and a more intact vasculature after stroke. We also established that pericytes do not contribute as largely to scar formation in the brain as previously suggested.

This thesis highlights the critical role of pericytes after stroke and identifies RGS5 as an important modulator of pericyte-related protection and recovery mechanism. Pericytes thus constitute an important target cell to develop new therapies for stroke.

# Populärwissenschaftliche Zusammenfassung

Ein Hirnschlag kann zu jeder Zeit passieren und es kann jeden treffen. Ein Hirnschlag kann das Leben einer Person, sowie das seiner Familie, von einer Sekunde auf die andere verändern.

Ein Hirnschlag, auch Schlaganfall genannt, ist eine dramatische Krankheit, die in den meisten Fällen von einem Blutgerinnsel ausgelöst wird. Dieses Gerinnsel unterbricht den Blutfluss in gewissen Teilen des Gehirns. Dadurch sterben die Zellen in diesem Gebiet ab. Je nach Länge und Lokalisierung des Hirnschlags haben die Patienten Lähmungserscheinungen, Sprachprobleme oder können sogar sterben. Einige Patienten erholen sich nach einem Hirnschlag vollständig, die Mehrheit jedoch lebt für immer mit dessen Nachwirkungen. Deshalb überrascht es wenig, dass der Hirnschlag nicht nur eine der häufigsten Todesursachen weltweit ist, sondern auch einer der gängigsten Gründe für Beeinträchtigungen. Die einzige Therapie, die es heutzutage gibt, beinhaltend die sofortige Entfernung des Blutgerinnsels, so dass der Blutfluss wiederhergestellt wird. Jedoch sind nur etwa 10% aller Patienten für diese Therapie qualifiziert. Daher wird intensiv nach neuen therapeutischen Alternativen geforscht.

Damit neue Therapien entwickelt werden können, müssen alle Prozesse, die nach einem Hirnschlag passieren, verstanden werden. Ein Hirnschlag führt zu einer Sauerstoffreduktion im betroffenen Gehirngebiet, aufgrund dessen die Gehirnzellen, sogenannte Neuronen, absterben. Zusätzlich wird die Bluthirnschranke durchlässiger. Die Bluthirnschranke kontrolliert welche Substanzen vom Blut ins Gehirn gelangen und sorgt dafür, dass keine giftigen Stoffe in Kontakt mit den sensiblen Neuronen kommen. Einer der Zelltypen, der die Bluthirnschranke bildet, sind die sogenannten Pericyten. Pericyten wickeln sich um die Blutgefässe und haben weitere vitale Funktionen im Gehirn. Neben ihrer Rolle in der Bluthirnschranke tragen sie auch zur Bildung von neuen Blutgefässen und Narbengewebe bei; beides Prozesse, die nach einem Hirnschlag vorkommen.

Das Ziel dieser Doktorarbeit war zu untersuchen, wie Pericyten nach einem Hirnschlag reagieren und ob diese Reaktionen beeinflusst werden können, um den Schaden zu mindern oder den Heilungsprozess zu unterstützen.

Es zeigte sich, dass Pericyten einer der ersten Zelltypen sind, die nach einem Hirnschlag reagieren. Eine der wichtigsten Erkenntnisse war, dass Pericyten schon vor dem Zusammenbruch der Bluthirnschranke auf den Hirnschlag reagieren. Unsere Hypothese war deshalb, dass Pericyten ein idealer Zielzelltyp sind, um diesen Zusammenbruch zu verhindern. In einem weiteren Schritt erkannten wir, dass die Entfernung eines spezifischen Genes namens *rgs5* die Reaktion der Pericyten modulierte. Bei Mäusen, die kein RGS5 Protein produzieren, war die Anzahl von Pericyten um die Blutgefässe herum grösser. Dies stabilisierte die Bluthirnschranke, und reduzierte den Schaden nach einem Hirnschlag. Diese Mäuse hatten eine grössere Anzahl stabilerer Blutgefässe nach einem Hirnschlag. Interessanterweise zeigte sich, dass Pericyten nur geringfügig zur Narbengewebebildung beitragen.

Diese Doktorarbeit hebt die kritische Funktion von Pericyten nach einem Hirnschlag hervor. Ausserdem identifiziert sie RGS5 als einen wichtigen möglichen Angriffspunkt an Pericyten um neue Behandlungen zu entwickeln, die Schutz- und Heilungsmechanismen nach einem Hirnschlag verstärken.

# Abbreviations

| | |
|---|---|
| **α-SMA** | alpha-smooth muscle actin |
| **ABC** | Avidin-biotin complex |
| **Ang** | Angiopoietin |
| **BBB** | Blood-brain barrier |
| **BDNF** | Brain-derived neurotrophic factor |
| **CBF** | Cerebral blood flow |
| **CNS** | Central nervous system |
| **Coll-I** | Type I Collagen |
| **Coll-IV** | Type IV Collagen |
| **CSF** | Cerebral spinal fluid |
| **DAB** | 3,3-diaminobenzidine |
| **DAPI** | 4′,6-diamidino-2-phenylindole |
| **DTT** | Dithiothreitol |
| **ECM** | Extracellular matrix |
| **FN** | Fibronectin |
| **GDP** | Guanosine diphosphate |
| **GFAP** | Glial fibrillary acidic protein |
| **GFP** | Green fluorescent protein |
| **GPCR** | G-protein coupled receptor |
| **GTP** | Guanosine triphosphate |
| **HET** | Heterozygous |
| **HRP** | Horseradish peroxidase |
| **IHC** | Immunohistochemistry |
| **i.v.** | intravenously |
| **KO** | Knock-out |
| **MCA** | middle cerebral artery |
| **MMP** | Matrix metalloproteinase |
| **NeuN** | Neuronal nuclei |
| **NG2** | Neuron-glial antigen 2 |
| **NVU** | Neurovascular unit |
| **PBS** | Phosphate buffered saline |
| **PBS-TX** | Phosphate buffered saline containing 0.1% Triton X-100 |
| **PDGF-BB** | Platelet-derived growth factor BB |
| **PDGFRß** | Platelet-derived growth factor receptor beta |
| **PFA** | Paraformaldehyde |
| **PI3K** | Phosphoinositide 3-kinase |
| **PIMO** | Pimonidazole |
| **pMCAO** | permanent middle cerebral artery occlusion |
| **PDCLX** | Podocalyxin |
| **qPCR** | quantitative polymerase chain reaction |
| **RGS5** | Regulator of G-protein signaling 5 |
| **RIPA** | Radioimmunoprecipitation assay buffer |
| **RT** | Room temperature |
| **Shh** | Sonic hedgehog |
| **SDS-PAGE** | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| **SMC** | Smooth muscle cell |
| **TJ** | Tight junction |
| **tPA** | Tissue plasminogen activator |
| **TUNEL** | terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling |
| **VE-Cadherin** | Vascular endothelial cadherin |
| **VEGF** | Vascular endothelial growth factor |
| **WB** | Western blot |
| **WT** | Wild type |
| **ZO-1** | Zonula occludens-1 |

# Introduction

Stroke remains one of the leading causes of death worldwide [1,2]. It can either be caused by interruption of the blood flow by a clot (ischemic stroke, 80 % of the cases) or rapture of a blood vessel (hemorrhagic stroke, 20%) in the brain [1,2]. Depending on the location of the stroke, patients experience different neurological deficits. As these symptoms can be permanent, stroke also comprises one of the most common causes of disability worldwide. An aging population, an increasing prevalence of risk factors, and a reduction in case fatality will further increase the burden of stroke in the decades to come [2].

Reestablishment of the blood flow by mechanical thrombectomy or thrombolysis with tissue plasminogen activator (tPA) has significantly improved the outcome after ischemic stroke [3-5]. However, the short time window and the risk of hemorrhagic bleeding allows only around 10% of stroke patients to be eligible for these, currently only available, interventions [6-8].

Thus, there is an increasing need to develop novel treatments for stroke. Despite several promising preclinical studies, their translation into clinical usage has so far been disappointing [5,9,10]. Many studies have focused on direct neuroprotection or neuro-restoration, but it becomes increasingly evident that successful recovery after stroke will have to address the entire neurovascular system [11-14].

## Stroke pathology

In ischemic stroke, the occlusion of a blood vessel results in the interruption of oxygen and nutrient delivery to the affected brain regions. This is the start of a cascade of molecular and cellular events leading to an ischemic injury and neuronal cell death [7,15]. Although neurological dysfunction arises within seconds to minutes after the occlusion, the ischemic injury and cell death progress for up to days and weeks [7] (**Figure 1**).

## Acute phase of stroke

The high intrinsic metabolic activity and the large concentration of the excitotoxic neurotransmitter glutamate make the brain especially vulnerable to ischemic stroke [16,17]. Within the infarct core, the blood flow drops to below 20% of its baseline rate, resulting in a depletion of the ATP stores and the failure of energy metabolism [7]. Due to this energy failure, as well as the failure of ion pumps and re-uptake mechanisms, glutamate accumulates in the extracellular space. This accumulation of glutamate subsequently results in an excessive influx of calcium, sodium, and water into neurons and the production of oxygen radicals, ultimately resulting in neuronal cell death [17] (**Figure 1**).

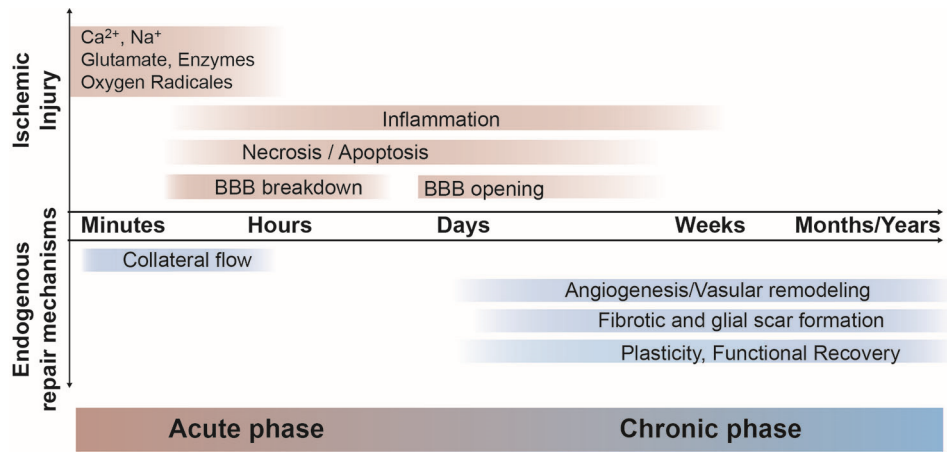


**Figure 1: Development of ischemic stroke pathology.**
Ischemic stroke occurs after the occlusion of a blood vessel. After this initial event, the pathology develops in different phases that can be divided into the acute and chronic phase. The ischemic injury triggers a cascade of events, including cell death and blood-brain barrier (BBB) breakdown. After stroke, several endogenous repair mechanisms are initiated, including vascular remodeling and scar formation. Based on [7,11,17,18].

The ischemic insult results in a breakdown of the blood-brain barrier (BBB) [19]. The timing of the BBB breakdown is debated. In stroke patients it has been shown that BBB breakdown can start within the first few hours after stroke [20]. This BBB breakdown is associated with increased permeability and vascular leakage, leading to the accumulation of blood-derived components and cells within the brain parenchyma. This further aggravates the brain damage after ischemic stroke [19,21,22]. Importantly, the BBB breakdown correlates with an increased risk of hemorrhagic transformation [20]. Hemorrhagic transformation is a complication occurring after a stroke, referring to a spectrum of ischemia-related brain hemorrhages that negatively impact on stroke outcome [6,23]. The increased risk of hemorrhagic transformation after delayed tPA treatment is one of the reasons for the small time-window for thrombolysis [3,22]. Preventing an early BBB breakdown, therefore, might

be an important approach to limit side-effects of tPA treatment and to prolong the therapeutic window and thereby enable more patients to receive treatment [20,22].

Within the first hours after stroke, an inflammatory response develops. Microglial cells and astrocytes are activated and release inflammatory cytokines and chemokines [24,25]. Additionally, endothelial and perivascular cells contribute to the cytokine production and upregulate the expression of adhesion molecules, which promotes leukocyte trafficking through the vessel wall [26]. The initial inflammatory response during the acute phase of stroke amplifies the ischemic injury; however, inflammation also promotes critical events necessary for tissue repair during the chronic phase after stroke [7].

## Chronic phase of stroke

Most stroke survivors show some degree of recovery over time. This recovery is due to several endogenous repair mechanisms that occur during the chronic phase after stroke. These repair mechanisms start days after the injury and are maintained for weeks. They include processes such as neural plasticity, vascular remodeling, and scar formation [27-29] (**Figure 1**).

Vascular remodeling re-establishes the blood flow and energy supply to the hypoxic tissue after stroke [28,30]. Directly after the occlusion of the blood vessel, collateral blood flow plays an important role in maintaining regional cerebral blood flow (CBF), and the efficiency of collateral recruitment correlates to the stroke outcome [28,31]. In the chronic phase, increased blood flow is attributed to angiogenesis, the formation of new blood vessels through proliferating endothelial cells [30,32]. In stroke patients, vessel number and density correlate with survival time [33]. Vascular remodeling, therefore, has increasingly gained interest as a target to improve functional outcome after stroke [32,34,35]. However, angiogenesis is associated with an opening of the BBB, which is necessary for new vessels to form. Newly formed vessels are not mature yet, and consists of vascular structures with compromised integrity [36]. The second opening of the BBB due to angiogenesis is observed after several days, and hence the BBB breakdown is often described as biphasic [19,37]. The opening of the BBB contributes to the vascular leakage that initially occurred after the BBB breakdown. Hence, the BBB opening constitutes a challenge in promoting angiogenesis as a therapeutic target [35,38,39] .

Scar formation is a common response to tissue injury in most organs. Injured tissue is separated from healthy tissue to prevent extensive inflammation and is replaced with extracellular matrix (ECM) proteins [29,40]. In the chronic phase of stroke, resident reactive astroglia assemble around the infarct core to seal off the intact tissue from the damaged ischemic core by forming a glial scar [25]. Enveloped by this glial scar, a dense fibrotic scar develops, consisting of fibrous ECM proteins such as collagens and fibronectin (FN) [40]. Under physiological conditions, the

extracellular space contains very little fibrous ECM, but is rather composed of a network of proteoglycans, hyaluronans, tenascins and link-proteins, which not only provide mechanical support, but also serve as a substrate for the compartmentalization of the extracellular space and function as a scaffold during development and adult neurogenesis [41-43]. The increased deposition of fibrous ECM proteins after stroke resulting in a stiff fibrotic scar is suggested to impede the anatomical plasticity within the central nervous system (CNS) and therefore impact negatively on learning and memory [40]. Therefore, the formation of these scars needs to be tightly regulated, as on one hand scar formation impacts negatively on functional recovery; but on the other hand, inhibiting scar formation within the CNS can have severe effects as well [44,45].

### The search for new therapeutic targets

The mechanisms leading to neuronal cell death have been extensively studied. Despite promising preclinical studies aiming at neuroprotection by preventing excitotoxicity, oxidative stress, inflammation, or apoptosis, their translation into the clinic has failed so far, as none of them have resulted in improved outcome in stroke patients [5,9,10].

One of the possible explanations for the failure of these trials could be their narrow focus on neurons [7,17]. It becomes increasingly evident that protecting or replacing neurons alone is not sufficient to treat stroke. Recent studies resulted in a shift from purely neuron-centric approaches to the recognition that successful neuroprotection and restoration are only feasible through targeting the entire neurovascular system [11-14]. However, there is a lack of understanding of how the neurovascular system and more specifically, the neurovascular unit, is affected after a stroke. This knowledge is crucial in finding new targets to protect and restore the neurovascular system, and with that, to treat stroke.

## The neurovascular system

The brain has one of the highest oxygen consumptions of our body, and to supply the brain with enough blood, the brain contains approximately 600-700 km of blood vessels [46]. Already in the late 19$^{th}$ and early 20$^{th}$ centuries, scientists discovered that the vasculature of the CNS is distinct from the vasculature in the rest of the body. Studies by Goldmann, among others, showed that injected dyes in the blood do not stain most parts of the brain, while those injected into the cerebral spinal fluid (CSF) stain only the brain [47]. This barrier separating the CNS from the circulation was later termed the *blood-brain barrier*. Nowadays, we know that the BBB regulates the uptake of water-soluble nutrients, metabolites, and molecules into the CNS, and is

composed of endothelial cells that are connected by tight junctions (TJs), pericytes, astrocytic end-feed and the basement membrane [48-50]. Together with the glial cells of the brain (astrocytes, microglia, and oligodendrocytes) and neurons, they form the neurovascular unit (NVU), which is necessary for the functional homeostasis within the brain [51,52].

# Pericytes

In the 1870s, Carl Joseph Eberth and Charles Rouget described a contractile cell that was located around endothelial cells of capillaries, which was later named pericyte by Zimmermann [53,54]. Despite being first described almost 150 years ago, most of our understanding of pericytes has been acquired in the last decades.

## Definition of pericytes

Since their discovery, it has remained challenging to establish a definition and appropriate identification criteria for pericytes, which is mainly due to the lack of a single marker identifying all pericytes [55-57]. Contributing to these difficulties may also the different developmental origins of pericytes. Accordingly, studies in chick-quail chimeras and transplantation studies have shown that pericytes in the forebrain originate from neural crest cells, while pericytes in the midbrain, hindbrain, and periphery mainly are mesoderm-derived [58-64]. However, even within the same tissue, pericytes can have different origins [65].

Today, the most commonly accepted definition of a pericyte includes its location and morphology, in combination with a series of histological markers (**Figure 2**). In brief, pericytes are perivascular cells lining the abluminal side of capillaries and are embedded within the vascular basement membrane.

*Location*

The vascular tree of the cerebrovascular system can be divided into several sections, with different compositions of mural cells, basement membranes, and functions [66]. Pericytes are located on pre-capillary arterioles, capillaries, and post-capillary venules, while smooth muscle cells (SMCs) are mainly found on arterioles and venules [56,66,67]. Pericytes are located in the center of the NVU, lining the abluminal side of several endothelial cells, and are embedded within the vascular basement membrane and astrocytic end-feet [51,52,68]. Furthermore, they are connected to endothelial cells through peg-socket contacts, integrins, and cell adhesion molecules [69].

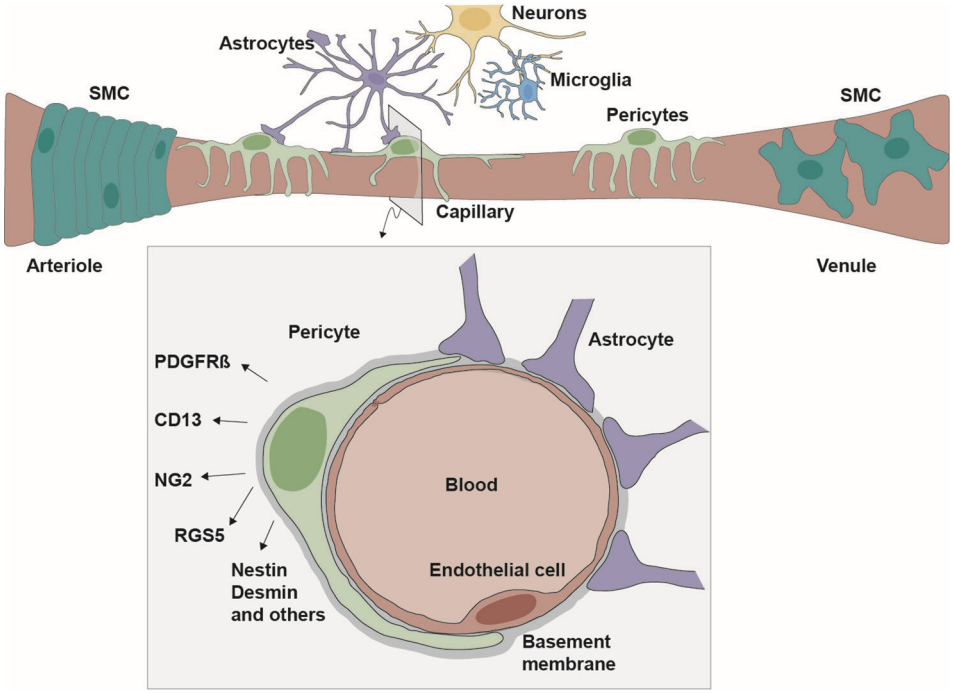


**Figure 2: Pericytes in the neurovascular unit.**
Pericytes (green) are located around capillaries, while smooth muscle cells (SMC, dark green) are found on arterioles and venules. Pericytes are part of the neurovascular unit, composed of endothelial cells (red), astrocytic end-feet (violet), neurons (yellow), and microglia (blue) and are embedded within the basement membrane (grey). They wrap around capillaries in the brain, and they express the markers PDGFRß, CD13, NG2, RGS5, and others. Based on [52,67,68].

*Morphology*

Pericytes have a round nucleus, in contrast to the elongated cigar-shaped nucleus of endothelial cells [70]. Additionally, pericytes extend their processes along capillaries. These processes can have different morphologies, depending on the vascular bed and the differentiation/developmental state, resulting in varying pericyte coverage [70]. Nevertheless, the brain and the retina have the highest pericyte coverage and pericyte-to-endothelial cell ratio (1:3) of the entire body [71]. The most common morphology is represented by a pericyte encircling the capillary with broad and continuous projections, resulting in a large area covered. Pericytes can change their morphology upon injury or migration, where they adopt a bulging cell body or a bipolar morphology, respectively.

*Markers and respective pathways*

One of the challenges in studying pericytes is the lack of a single marker to identify them. Pericytes can express different markers throughout their development and maturation states [56,72]. Also, in response to injury, they up- or downregulate specific markers. During the course of this thesis, the following pericyte markers were utilized:

Platelet-derived growth factor receptor beta *(PDGFRß)* is a tyrosine kinase receptor and expressed on pericytes [73,74]. Platelet-derived growth factor (PDGF)-BB, which is secreted by endothelial cells, binds with high affinity to PDGFRß [75]. Precise spatial regulation of PDGF-BB is achieved through a retention motif, a positively charged C-terminus that binds to negatively charged heparin sulfate proteoglycans within the ECM [76]. Upon binding of PDGF-BB to PDGFRß, homodimerization of the receptor occurs, leading to its autophosphorylation on several tyrosine residues and internalization of the receptor [77,78]. Depending on the phosphorylation site, a variety of downstream pathways are activated, resulting in pericyte proliferation, migration, survival, and recruitment to the vessel wall [52,79]. Mice with interrupted PDGFRß-signaling have a substantially reduced pericyte coverage, and complete loss of either PDGF-BB or PDGFRß is embryonically lethal due to severe hemorrhage [74-76,80].

Alanyl aminopeptidase (*CD13*) is a type II membrane zinc-dependent metalloprotease that has been described as a marker for cerebral pericytes [81-83]. CD13 has been suggested to be involved in angiogenesis, as CD13 is essential for capillary tube formation and degradation of the vascular basement membrane protein type IV collagen (Coll-IV), allowing for the sprouting of new blood vessels [84,85].

Chondroitin sulfate proteoglycan 4/Neuroglial antigen 2 (*NG2*) is an integral membrane chondroitin sulfate proteoglycan [86]. Pericytes increase the expression of NG2 during developmental and pathological conditions such as stroke and cancer [87,88]. Therefore, NG2 has been described as a marker for an activated state of pericytes in response to vascular changes during angiogenesis, vessel stabilization, and vascular remodeling. Mice lacking NG2 have lower pericyte coverage and reduced angiogenesis [87]. NG2 has also been used as a marker for mature pericytes [89,90]. Therefore, NG2 expression in pericytes may be important for vascular remodeling as well as vessel stabilization.

Regulator of G-protein signaling 5 (*RGS5*) is a negative regulator of G-protein coupled receptor (GPCR) signaling [91-93]. GPCRs represent the largest group of membrane receptors in eukaryotes, and upon ligand binding, their Gα proteins exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP), resulting in its dissociation from the Gβγ subunit, after which both subunits autonomously activate downstream signaling pathways (**Figure 3**). GPCR signaling is tightly controlled by, among others, RGS proteins. RGS proteins act as GTPase-

activating proteins, and interact specifically with the GPCR Gα subunits and accelerate their GTPase activity resulting in a timely signal termination [91,93]. RGS5 belongs to the B/R4 group of RGS proteins and specifically binds to the $G\alpha_{i/o}$ and $G\alpha_q$ subunits of GPCRs. It shares sequence homologies with other B/R4 family members, particularly RGS4 and RGS16 [91]. Further, the mRNA of human and mouse RGS5 is 90% identical, indicating important biological functions that are evolutionarily conserved [94]. RGS5 was first described as a brain pericyte marker by Bondiers *et al.*, when they found that RGS5 is among the most downregulated genes in PDGFRß-KO embryos [95]. RGS5 has been shown to be highly expressed during development when vessels acquire a pericyte coverage [92,96]. Interestingly, the vasculature of RGS5-deficient mice develops normally and with no alterations in pericyte numbers, indicating that developmental neovascularization is not dependent on RGS5 [97,98]. However, it has been shown that RGS5 is upregulated in response to several neurological conditions, including stroke, as well as in various tumor types [99-104]. RGS5 has been associated with angiogenesis and studies in RGS5-KO mice have suggested its involvement in pericyte maturation, vascular remodeling, stabilization, and normalization [96,105-108]. Further, RGS5 expression is increased in arteriogenesis through nitric oxide [106]. After stroke, it has been shown that pericytes upregulate RGS5, and pericytes that detach from the vessel wall express RGS5, indicating that RGS5 may regulate pericyte detachment [99].

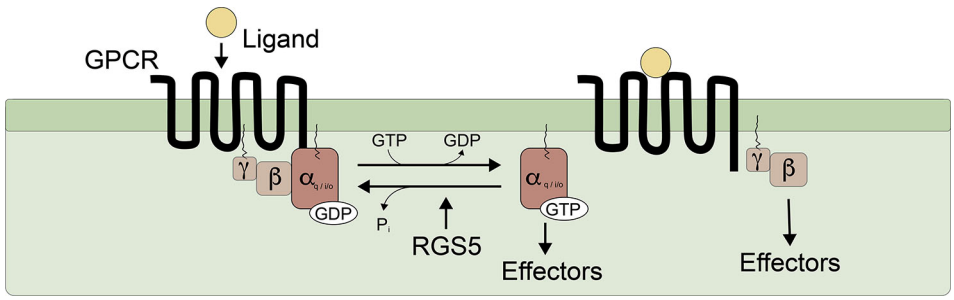


**Figure 3: G-protein coupled receptor signaling.**
Upon binding of a ligand to a G-protein coupled receptor (GPCR), guanosine diphosphate (GDP) on the Gα subunit is exchanged to guanosine triphosphate (GTP), and the Gα subunit dissociates from the Gβγ subunit. RGS5 acts as a GTPase, and therefore is a negative regulator of GPCR-mediated signaling.

Other pericyte markers that were not used in this thesis include the structural and filament markers Desmin and Nestin [109-111]. While pericytes on pre-and post-capillaries express alpha-smooth muscle actin (α-SMA), capillary pericytes have been described to only express α-SMA upon culturing *in vitro* [67,112,113]. Recent advantages in single-cell transcriptomics shed even more information onto the complex expression pattern of pericytes [114,115]. Using different cell isolation approaches, Vanlandewijck *et al.* found that the transcriptome of pericytes is homogenous, while Zeisel *et al.* described three different pericyte clusters [114,115].

Despite this discrepancy, these studies characterized pericytes on a transcriptional level, and further, highlighted the importance on how different isolation and selection criteria can influence study results.

## Functions and dysfunction of brain pericytes

Pericytes have multiple roles within the brain (**Figure 4)**. Therefore, loss of pericytes or pericyte dysfunction have substantial negative impact on brain homeostasis.

*Blood-brain barrier formation and maintenance*

Due to their strategic location within the NVU, pericytes are a crucial part of the BBB. During embryonic development of mice, nascent "leaky" vessels are formed around E10 [116,117]. Through the activation of Wnt/ß-catenin signaling, important genes are switched on that induce the formation of a primitive BBB by day E15 [118,119]. Pericytes are recruited to the nascent vessels during this step and regulate the formation of endothelial TJs as well as trans-endothelial trafficking [120]. Pericytes are crucial in the formation of the BBB, as *pdgfrß*$^{-/-}$ mice are embryonically lethal due to pericyte-loss-induced microaneurysms [74,120]. Whether humans are born with a functional BBB remains unknown [121]. During adulthood, pericytes continue to be crucial in maintaining and regulating the BBB integrity [50,122]. They control the expression and alignment of tight and adherent junctions, as well as transcytosis across the BBB [50,122]. Studies performed in adult viable pericyte-deficient mice have shown that a reduction in pericyte numbers leads to increased permeability of the BBB, both through transcytosis and paracellular pathways and, in an age-dependent fashion, leading to the disruption of endothelial TJs [50,122]. Also, a recent study showed that inducing pericyte loss in adult mice is sufficient to initiate BBB breakdown [123].

*Angiogenesis*

Pericytes play a significant role in regulating angiogenesis during development as well as during vessel remodeling [56,68,124]. Angiogenesis is a process where new vessels are formed by sprouting from existing vessels. This can be divided into three major steps: (i) the initiation of angiogenesis, (ii) sprout formation, migration, and stabilization, and (iii) maturation and termination [39,124,125]. The importance of pericytes in angiogenesis is reflected by the fact that they participate in all steps of angiogenesis.

Upon pro-angiogenic stimuli, endothelial cells become motile and form tip cells, which together with pericytes start expressing matrix metalloproteinases (MMPs) to degrade the basal lamina [126,127]. This enables pericytes to detach and to liberate the tip cells further. Endothelial cells form stalk cells that proliferate and form a new vessel tube [128,129]. Once the angiogenic sprouts are formed, pericytes are recruited

to the newly formed blood vessels through endothelial cell-derived PDGF-BB [75,76,130]. Subsequently, pericytes stabilize the new vessel and regulate the deposition of vascular basement membrane proteins [131,132]. Pericytes also inhibit endothelial cell proliferation and facilitate endothelial cells returning to a quiescent state [131,133]. Therefore, pericytes are crucial in vessel stabilization and maturation. Pericyte-deficient mice have a reduction in angiogenesis both during development and adulthood [76,130,134].

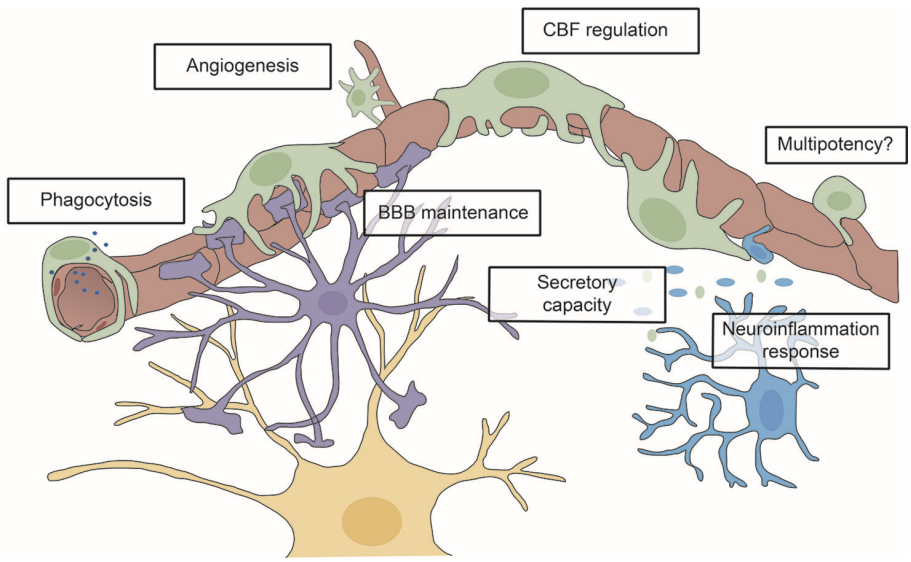


**Figure 4: Diverse functions of brain pericytes.**
Pericytes fulfill multiple roles in the brain. These functions include BBB maintenance, angiogenesis, phagocytosis, neuroinflammatory response, cerebral blood flow (CBF) regulation, and potential multipotent functions. Based on [15,52].

*Secretory capacity*

In response to microenvironmental cues, pericytes secrete a broad range of molecules [135,136]. Depending on tissue and stimuli, they secrete pro- and anti-inflammatory factors, cytokines, chemokines, growth factors, and ECM, and thus, the pericyte secretome plays an essential part in inflammation, angiogenesis, and tissue regeneration. Recently, our group has shown that pericytes also shed microvesicles from their plasma membrane [137]. The pericyte secretome is not only essential for normal brain homeostasis, but likely contributes to the progression of several pathologies or can be exploited to stimulate regeneration [135,138].

*Other pericyte functions*

Pericytes have a number of other important functions that have, however, not been examined in this thesis.

Cerebral blood flow (CBF) regulation: Pericytes are suggested to regulate the capillary tone and diameter, as well as to constrict after injury, thereby impairing the reflow of blood [139-141]. However, the apparent lack of α-SMA expression in capillary pericytes, as well as deviations from the commonly accepted pericyte definition, has resulted in a controversy regarding the contribution of pericytes to the regulation of the CBF [66,142].

Neuroinflammatory response: Pericytes have been described to contribute to the neuroinflammatory response and regulate leukocyte trafficking [143-145]. *In vitro*, it has been shown that pericytes influence neuroinflammation and both respond to and secrete inflammatory molecules and cytokines [135,136,143,146].

Clearance: Due to their ability to take up a variety of small soluble molecules and to clear toxic circulating plasma proteins as well as cellular debris, pericytes have been proposed to be important in the clearance of the brain [56,147,148]. Additionally, their phagocytotic capacity is increased during neuroinflammation [146]. Their clearance function might be impaired in different pathologies, including neurodegenerative disorders [149].

Multipotency: Multiple *in vitro* studies have shown that pericytes have the capability to differentiate into a variety of cell types, including neuronal and glial like lineages [110,150,151]. However, one study using lineage tracing stated that pericytes do not contribute to other cell lineages *in vivo* during aging nor in several pathological conditions [152].

# Pericytes in stroke

Due to their multiple functions and their strategic position in the center of the NVU, pericytes have been suggested to play a crucial part in the stroke pathology [153,154] .

## Pericyte constriction and death

Pericytes are vulnerable to ischemic injury [154]. Early work performed using electron microscopy in spontaneous hypertensive stroke-prone rats has suggested the presence of two different subtypes of pericytes, granular and fibrous pericytes [155]. Granular pericytes have been shown to grow in size while filamentous pericytes degenerated during the development of hypertension before the rats developed stroke symptoms.

After stroke and simulated ischemia in brain slice cultures, it has been shown that pericytes constrict and die around the blood vessels [141,156]. Pericyte constriction depends on intracellular calcium concentrations, which is disturbed due to energy failure after stroke [139,157]. Calcium influx in neurons contributes to their early cell death; however, pericyte cell death has only been studied to a limited degree [156,158]. In particular, the timing of pericyte death as well as whether specific subpopulations are more susceptible to cell death remains unknown.

## Pericyte detachment

Historically, one of the first pieces of evidence indicating that pericytes respond directly to stroke was provided by Gonul *et al.*, showing that pericytes form peaks to migrate as early as 2h in a cat model of stroke [159]. This detachment was followed up later in rats, showing that the space between pericytes and endothelial cells is increased at 3h after stroke [160].

Further, the detachment of pericytes has been suggested to be dependent on RGS5, as pericytes that detach from blood vessels express RGS5 [99]. However, little else is known about the role of RGS5 in stroke, especially whether the deletion of *rgs5* has an impact on the detachment of pericytes and thereby can provide a possible therapeutic target.

## Pericyte-related blood-brain barrier dysfunction

Pericyte loss has been suggested to contribute to BBB breakdown after stroke [153,161,162]. This is based on observations that pericyte-deficiency leads to impairment of the BBB integrity under physiological conditions, as well as during development and in several CNS diseases [50,120,122,162,163].

However, few studies have directly addressed the causal link between pericyte loss and BBB breakdown after stroke [164-166]. These studies showed that postnatally induced systemic PDGFRß knockout (KO) mice have reduced SMA-$\alpha^+$ pericyte coverage, which is associated with increased vascular leakage as well as decreased and deformed TJ proteins at 1 day after photothrombotic stroke [164,165]. Additionally, angiopoietin (Ang)-2 gain-of-function mice, which have reduced pericyte coverage under physiological conditions, have increased vascular leakage at 24h after transient stroke [166]. However, whether pericyte loss, either through cell death or detachment, precedes BBB breakdown in wild-type mice is unknown. Hence, whether pericytes can be a target to prevent BBB breakdown, and whether this protection can be achieved by increasing pericyte numbers or blocking their detachment, remains elusive.

## Angiogenesis and vascular remodeling

Angiogenesis and vascular remodeling are necessary to re-supply the ischemic tissue with oxygen and nutrients, and are therefore crucial for tissue preservation and restoration [30]. As mentioned above, increased perfusion and vessel density are beneficial in recovery after stroke; however, increasing angiogenesis harbors the problem of enhanced BBB leakage [33-35,167].

As critical modulators of angiogenesis, pericytes play an important role in angiogenesis and vascular remodeling after stroke. Several angiogenesis-related pathways are activated in response to stroke that depend on pericytes. Accordingly, pericytes have been shown to increase the secretion of the proangiogenic factor vascular endothelial growth factor (VEGF)-A in response to hypoxia [168]. VEGF-A further induces the upregulation of Ang1 and Tie-2 in pericytes [169]. Endothelial cell sprouting begins within the first 24h hours after stroke, and new vessels are formed within days [30,170,171]. PDGFRß, which is required to recruit pericytes to immature vessels, is upregulated following stroke and disturbances in PDGFRß-signaling after stroke result in increased vascular leakage due to reduced pericyte recruitment to immature vessels [165,172-174]. However, whether improved vascular remodeling and maturation of immature vessels could be achieved by targeting the pericyte response is unknown.

RGS5 has been described as an angiogenic marker of pericytes, that during development, is induced in a HIF-1α dependent manner [105]. In the hypoxic environment of tumors, it has been shown that RGS5-deficiency results in pericyte maturation, contributing to vessel normalization [107]. This indicates its relevance in hypoxic environments in the adult.

However, little is known about the role of RGS5 after stroke. Understanding how RGS5 might affect pericyte maturation and vascular remodeling could be an essential step in developing novel therapeutic approaches to stabilize newly formed vessels after stroke.

## Scar formation

Following a stroke, there is an increase in PDGFRß$^+$ cells within the infarct core, suggested to occur both by the migration of cells into the infarct core, as well as by the proliferation of the resident PDGFRß$^+$ cells [158,165,174]. Interestingly, some of these PDGFRß$^+$ cells migrate away from the blood vessels and remain in the parenchyma. It has been suggested that these parenchymal PDGFRß$^+$ cells participate in the formation of the fibrotic scar by depositing ECM proteins, such as type I collagen (Coll-I) and FN [158,175]. Fibrosis has been well studied in a variety of organs, including liver and kidney, and pericytes are suggested to be the main source of scar-forming cells in those tissues [176,177]. However, the fibrotic scar after stroke is

relatively poorly studied, and in contrast to other organs, there is a lack of lineage tracing studies confirming the extent of the pericytes' contribution to scar formation. Studies in the spinal cord suggest that targeting pericytes might alter the fibrotic scar [178,179]. Whether targeting pericytes after stroke has an impact on the formation of the fibrotic scar remains to be investigated.

The pericyte-astrocyte crosstalk under physiological conditions would suggest that pericytes might influence the formation of the glial scar as well, but again, little is known also in this regard [50,180].

## Other pericyte-related events after stroke

There are several other functions of pericytes in stroke. An increase in granular pericytes with the capacity to accumulate injected lipid components has been observed within the first few hours after stroke, indicating a phagocytic capacity of pericytes [159,181]. Pericytes might also modulate the inflammatory response after stroke through their secretome [136]. Additionally, a subpopulation of pericytes has been described to acquire a microglial phenotype, supporting an immune-regulating function of pericytes after stroke [99]. Further contributing to inflammation after stroke is the infiltration of cells from the periphery, and due to their function in mediating leukocyte trafficking, pericyte loss after stroke might impact on the infiltration of peripheral immune cells [136,143-145].

Despite the growing interest in the protection of the NVU, the role of pericytes after and their response to stroke remains rather unclear. As indicated above, pericytes are involved in a number of important processes after stroke, and thus might be a valuable target for novel stroke therapies. However, to develop new therapeutic approaches, it is crucial to know the timeline of events after stroke and whether one can achieve neurovascular protection and recovery by targeting brain pericytes.

# Aims of the thesis

The overall goal of this thesis was to study the role of pericytes after ischemic stroke. The specific aims were to:

1) establish a timeline of the pericyte response after stroke in relation to endothelial cells and the BBB breakdown (**Paper I**).
2) target the pericyte response by deletion of *rgs5* and investigate whether loss of RGS5
    a) affects BBB breakdown in the acute phase after stroke (**Paper II**).
    b) impacts on vascular stabilization in the chronic phase after stroke (**Paper III**).
    c) influences the scar formation after stroke in the chronic phase after stroke (**Paper IV**).

# Material and Methods

## Ethical considerations

All animal experiments of this thesis were approved by the Ethical Committee of Lund University, and methods were carried out in accordance with the relevant guidelines and regulations.

Animals were housed under standard conditions with a 12h light/dark cycle and had access to food and water *ad libitum*.

## Animals

In this thesis, several mouse strains were used, and all experiments were performed on male mice aged 8-12 weeks.

In **Paper I**, wild-type C57bl/6 mice were used. In **Papers II-IV**, we utilized a knock-out/knock-in reporter mouse strain, that expresses green fluorescent protein (GFP) under the promoter of RGS5 in a C57bl/6 background [98]. In particular, we used *rgs5*$^{gfp/gfp}$ mice (referred to as RGS5-KO) and wild-type mice (*rgs5*$^{+/+}$, referred to as WT) as control mice. To visualize activated pericytes, we used *rgs5*$^{gfp/+}$ (referred to as RGS5-HET) as a control (**Papers I-III**). In RGS5-HET mice, one of the alleles of RGS5 is replaced by GFP, making it possible to track pericytes by GFP-expression under the activated RGS5 promotor. In RGS5-KO mice, both alleles of RGS5 are replaced by GFP, whereby only GFP is expressed upon RGS5 promotor activity, but no RGS5 protein is produced.

RGS5-KO mice have previously been extensively validated and characterized and shown to be viable, fertile, and to develop without apparent defect [98,99]. RGS5 has been shown to be expressed in brain pericytes and in SMC [98,114,115]. However, we have previously shown that GFP is expressed in brain pericytes located on capillaries, and not in $\alpha$-SMA$^{+}$ cells [99]. Under physiological conditions, the pericyte number and vascular densities are not changed between RGS5-KO mice and WT mice (**Paper II**).

# Animal model of stroke

There are several different experimental stroke models available. In this thesis, we used a permanent experimental stroke model, in which the distal part of the left middle cerebral artery (MCA) was permanently occluded to induce a focal cerebral ischemia [182] (**Figure 5**). This stroke model is characterized by a high reproducibility and low mortality. For all surgeries, animals were kept on a heating pad and were anesthetized with isoflurane. An incision was made between the left ear and eye. The temporal muscle was detached from the skull in its apical and dorsal parts. A small craniotomy was made with a surgical drill above the anterior distal branch of the MCA, located in the rostral part of the temporal area, dorsal to the retro-orbital sinus. After exposure, the MCA was permanently occluded by electrocoagulation using an electrosurgical unit (ICC50; Erbe). For pain relief, Marcain (AstraZeneca) was locally applied, and the wound was sutured. Sham-operated animals were treated the same way, but without ligation of the MCA.

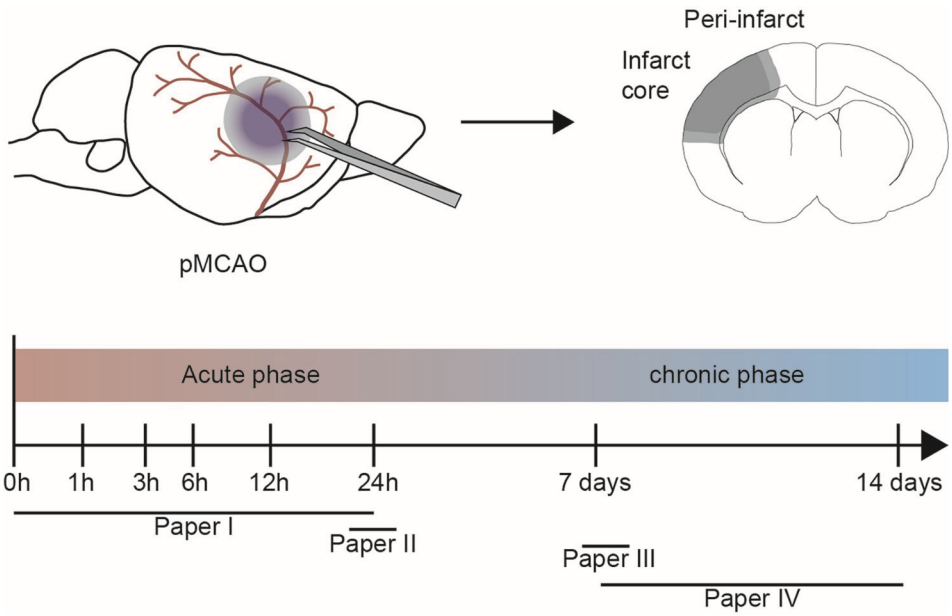


**Figure 5: Overview of the experimental setup.**
We permanently occluded the middle cerebral artery (pMCAO), which resulted in a cortical stroke, with an infarct core demarcated by a peri-infarct area. Papers I and II used timepoints in the acute phase after stroke, while Papers III and IV investigated timepoints in the chronic phase.

# Tissue collection

For the different studies, mice were sacrificed at either 1h, 3h, 6h, 12h and 24h (**Paper I**), at 24h (**Paper II**), at 7 days after stroke (**Papers III** and **IV**) and at 14 days after stroke (**Paper IV**) (**Figure 5**). For immunohistological analysis, mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brains were removed and placed in 4% PFA and postfixed overnight. They were then placed in 30% sucrose in PBS for 24h and sectioned in coronal sections of 40 µm in 12 series. For Western blot (WB) and qPCR analysis, mice were transcardially perfused with PBS, and the infarct area and corresponding area on the contralateral sides and in sham animals were dissected and frozen at -80°C until further processing.

# Blood-brain barrier leakage assessment

We used several methods to assess different degrees of vascular leakage after stroke.

In **Papers I** and **III**, endogenous fibrinogen staining was used to determine the accumulation of blood-derived fibrinogen by immunohistochemistry (IHC, see details further down). Fibrinogen is a large molecule (around 340 kDa), indicating that the BBB is permeable for large sized molecules.

In **Paper III**, we used the azo dye, Evans blue (Sigma-Aldrich), which binds to serum albumin (around 67 kDa), to investigate the vascular leakage. For this, 0.1 ml 2% Evans blue was injected into the tail vein 2h prior to termination. Mice were transcardially perfused with saline, and brains were stripped on ice and weighed. Each sample was homogenized in 25% trichloroacetic acid solution, kept at 4°C overnight, and then centrifuged for 30 minutes at 1000 x g at 4°C. The Evans blue content in 100 µl supernatant was measured at 620 nm using a 96-well plate reader. All values were within the standard curve consisting of diluted Evans blue in 1x PBS in the range from 1 to 100 ng/ml (R=0.98).

To assess subtle leakage, a 3 kDa fluorescent-labeled Dextran (Thermo Fisher Scientific) was injected intravenously (i.v.) 30 minutes prior to termination (**Paper I**). In **Paper II**, a 10 kDa fluorescent-labeled Dextran tracer was injected 15 minutes before termination. Mice were perfused as described above, and extravasation was analyzed with confocal microscopy. As controls, sham animals were injected with Dextran tracers and stroke mice with PBS, respectively. Animals were perfused with 4% PFA as described above.

# Immunohistochemistry

For IHC, brain sections were washed 3 times in PBS for 5 minutes and then blocked for 1h in 5% normal donkey or goat serum in 0.25% Triton X-100 (Alfa Aesar) in PBS (PBS-TX). Primary antibodies (**Table 1**) were incubated overnight at room temperature (RT) in 3% serum in PBS-TX. For PDGFRß detection, sections were pretreated with citrate buffer for 20 minutes at 80°C.

For immunofluorescence, sections were washed with PBS, and the staining was visualized using species-specific fluorophore-conjugated or biotin-conjugated secondary antibodies followed by fluorophore-conjugated streptavidin (Invitrogen). The nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000).

For brightfield staining, sections were quenched with a peroxidase solution (3% $H_2O_2$, 10% methanol, diluted in PBS) for 15 minutes before blocking. After incubation with the primary antibody, sections were incubated for 2h with corresponding biotinylated secondary antibodies (1:200, Vector Laboratories), followed by 1h signal enhancement with an avidin-biotin kit (Vectastain Elite ABC kit, Vector Laboratories) and revealed using chromogen 3,3-diaminobenzidine (DAB, Peroxidase Substrate Kit, Vector Laboratories).

**Table 1. List of primary antibodies used in this thesis.**
IHC: immunohistochemistry; WB: western blot; Coll-I: Type I Collagen; Coll-IV: Type IV Collagen; GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; NeuN: neuronal nuclei; NG2: neuron-glia antigen 2; PDCLX: Podocalyxin; PDGFRß: platelet-derived growth factor receptor beta; ZO-1: Zonula occludens-1

| Antibody | Species | Company | Catalog number | Dilution IHC | Dilution WB | Paper used |
|---|---|---|---|---|---|---|
| Aquaporin-4 | rabbit | Millipore | AB2218 | 1:1000 | | II |
| CD13 | rat | AbD Serotec | MCA2183 | 1:100 | | I, II, III |
| Claudin-5 | rabbit | Abcam | ab15106 | 1:1000 | 1:1000 | II |
| Coll-I | rabbit | Rockland | 600-401-103-0.5 | 1:400 | | IV |
| Coll-IV | rabbit | AbD Serotec | 2150-1470 | 1:500 | | IV |
| Fibrinogen | rabbit | Abcam | ab27913 | 1:400 | | I, III |
| FN | mouse | BD Biosciences | 610077 | 1:400 | | IV |
| GFAP | rabbit | Abcam | ab7260 | 1:400 | | IV |
| GFP | chicken | Abcam | ab13970 | 1:5000 | | I, II, III |
| Ki67 | rabbit | Abcam | ab15580 | 1:400 | | I |
| Laminin | rabbit | Abcam | ab11575 | 1:400 | | IV |
| NeuN | mouse | Millipore | MAB377 | 1:500 | | I, II, III, IV |
| NG2 | rabbit | Millipore | AB5320 | 1:200 | | I, II |
| PECAM-1 (CD31) | rat | R&D Systems | AF3628 | 1:400 | | I, II, III, IV |
| PDCLX | goat | R&D systems | AF1556-SP | 1:400 | | I, III, IV |
| PDGFRß | rabbit | Cell Signaling | 3169S | 1:200 | | I, II, III, IV |
| PDGFRß | rat | eBioScience | 14-1402-81 | 1:200 | | IV |
| PDGFRß | rabbit | Cell Signaling | 4564 | | 1:1000 | III |
| p-PDGFRß $_{Tyr751}$ | rabbit | Cell Signaling | 3161 | | 1:1000 | III |
| VE-Cadherin | rabbit | Abcam | Ab33168 | 1:1000 | 1:1000 | II |
| ZO-1 | rabbit | Fisher | 40-2300 | 1:500 | 1:500 | II |

# Imaging analysis

## Image acquisition

DAB stained sections were imaged using an Olympus BX53 light microscope equipped with the digital imaging software CellSense (**Papers I-III**).

Fluorescent immunostainings were visualized using an epifluorescence microscope system (Olympus BX53) (**Papers II- IV**), or a confocal microscope (Leica SP8 in **Papers I, III, IV**; Zeiss LSM510 and Zeiss LSM780 in **Paper II**).

## Quantification

For all IHC quantifications, 2-3 sequential sections per mouse were analyzed. For the quantification of cell numbers, the numbers were subsequently recalculated and reported as numbers per $mm^2$.

*Pericyte quantifications*

In brightfield images, pericyte numbers were counted according to their morphology and one pericyte marker. For confocal analysis, pericyte numbers were assessed by counting cells positive for a pericyte marker with a $DAPI^+$ nucleus and a perivascular location around capillaries (< 10 µm in diameter).

For pericyte coverage, pericytes and blood vessel stainings were separately subjected to threshold processing, and pericyte coverage was determined as the percentage of pericyte area covering the blood vessel surface. The total area covered by PDGFRß was analyzed using the ImageJ area measurement tool, where pictures were subjected to a threshold processing, which produced a binary image. The density was expressed as the percentage area of the total area analyzed.

Parenchymal and perivascular $PDGFRß^+$ cells were distinguished by their morphology and location in relation to blood vessels. $PDGFRß^+$ cells with a clear cell soma and processes around vascular structures were classified as perivascular $PDGFRß^+$ cells, while $PDGFRß^+$ cells located distant from the vessel with an amoeboid-like morphology and multipolar irregular cell projections were classified as parenchymal $PDGFRß^+$ cells.

*Blood vessel analysis*

Blood vessel density was analyzed using a vascular marker (CD31 or podocalyxin (PDCLX)) and the ImageJ area measurement tool. The density was expressed as the percentage of the area positive for a vessel marker of the total area analyzed. For the total vessel length, maximal projection images of a vessel marker were analyzed with ImageJ and reported as $µm/mm^2$.

*Vascular leakage analysis*

Extravascular fibrinogen and Dextran tracers were assessed by co-staining with a vessel marker. The blood vessel marker was used to subtract intravascular fibrinogen and Dextran within blood vessels. Afterwards, using ImageJ, the area covered by either fibrinogen or Dextran was analyzed and reported as the percentage of the total area analyzed.

*Hypoxia detection*

To detect hypoxia in the infarct area after stroke in **Paper II**, we applied the HypoxyprobeTM-1 kit (Hypoxyprobe, Inc.). Pimonidazole (PIMO) was injected to mice intraperitoneally (60 mg/kg) 60 minutes prior to perfusion. Afterwards, IHC was performed using the provided antibodies in the HypoxyprobeTM-1 kit. Quantification of the hypoxic area was performed using CellSens digital imaging software.

*Cell death assessment*

To assess cell death (**Papers I** and **II**), sections were first stained using the standard fluorescent staining protocol (as described above) for the desired cell types, and then incubated with terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) reaction mix (In Situ Cell death detection Kit, TMR red, Merck), according to manufacturer's instructions. Double-labeling with TUNEL was assessed by confocal microscopy.

*Stroke size assessment*

In **Papers I, III** and **IV**, stroke size was assessed using neuronal nuclei (NeuN) staining according to the standard immunohistochemistry protocol (described above). In **Paper II**, stroke size was analyzed using cresyl violet staining. For this, whole brain sections were mounted on glass slides and air-dried. After a short wash, they were immersed 2x3 minutes in 100% ethanol. The sections were immersed in 100% xylene for 15 minutes, followed by 10 minutes in 100% ethanol. Sections were rehydrated through alcohol (100%) 2x3 minutes and washed with water. Afterward, sections were placed in 0.1% cresyl violet for 5 minutes, before rinsing in water to remove excess stain. Sections were washed in 70% ethanol and dehydrated through 2x3 minutes in 100% ethanol, followed by 2x2 minutes in xylene.

Both NeuN and cresyl violet stained sections were cover-slipped and air-dried. Slides were scanned with a high-resolution scanner. In ImageJ, the areas of the contralateral hemisphere, ipsilateral hemisphere, and infarct area were outlined, and their areas were measured. The volume of infarct was calculated subsequently. Percentage of infarct volume was calculated as

$$= 100 \times \frac{V_{contra} \times V_{ipsi\ w/o\ infarct}}{V_{contra}}$$

and the percentage of swelling as

$$= 100 \times \frac{V_{ipsi} \times V_{contra}}{V_{ipsi}}$$

where $V_{ipsi}$ is the volume of ipsilateral hemisphere and $V_{contra}$ the volume of the contralateral hemisphere.

*Extracellular matrix deposition*

To assess ECM deposition as a readout for the fibrotic scar formation in **Paper IV**, Coll-I and FN staining were analyzed. The density was assessed as described above. To determine the contribution of PDGFRß$^+$ cells to ECM production, PDGFRß was used as a counterstain. Parenchymal and perivascular PDGFRß$^+$ cells were identified, and the number of cells double-labeled with Coll-I and FN were counted.

*Basement membrane analysis*

Coll-IV and laminin were used to visualize the vascular basement membrane. A blood vessel marker in combination with Coll-IV and laminin, respectively, was used to determine the thickness of the basement membrane using the measurement tool in ImageJ. For each measurement, the center of a capillary was determined, and the thickness was measured from the capillary wall to the outer edge of the vascular basement membrane. Measurements were repeated along capillaries at intervals of 5 µm.

*Glial scar analysis*

Glial fibrillary acidic protein (GFAP)$^+$ cells were used to analyze the development of the glial scar. The glial scar thickness was defined as the distance between the border of the infarct core and the outer border of the peri-infarct area delineated by hypertrophic GFAP$^+$ cells. GFAP$^+$ cells were counted using DAPI as a nuclear marker, and the density was assessed using ImageJ.

# Protein analysis by Western blot

For WB analysis, tissue was collected as described above and cut into small pieces. Two different protein isolation protocols were used in this thesis. For **Paper I**, the tissue was suspended in 2% sodium dodecyl sulfate (SDS) in Tris-HCl lysis buffer containing 1x protease and 1x phosphatase inhibitors (Thermo Fisher Scientific). Samples were then sonicated with a Q125 Sonicator (QSonica Sonicators) and

centrifuged for 10 minutes at 15000g at RT. For **Papers II** and **III**, tissue was suspended in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) with 1x protease and 1x phosphatase inhibitors (Thermo Fisher Scientific) and homogenized with Lysing Matrix D (MP Biomedical). For all papers, protein concentrations were evaluated with the Pierce BCA kit (Thermo Fisher Scientific). Samples were either supplemented to contain 0.1M dithiothreitol (DTT), 10% glycerol and 0.004% bromophenol blue (**Paper I**) or with Laemmli buffer (BioRad) containing ß-mercaptoethanol (**Papers II and III**) and heated to 95°C for 5 minutes. Equal amounts of protein (5 µg in **Paper I**, 50 µg in **Papers II** and **III**) were resolved on precast 4-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) TGX-gels (BioRad). Gels were transferred onto nitrocellulose membranes using the Trans-Blot turbo transfer system from BioRad. Membranes were blocked for 1h in either 5% w/v non-fat dry milk or 5% bovine serum albumin and then incubated with primary antibodies overnight at 4°C (see **Table 1** for a list of the antibodies used). After washing, species-specific horseradish peroxidase (HRP)-conjugated antibodies were revealed with Clarity Substrate (BioRad). Images were acquired using the ChemiDoc MP system (BioRad) and analyzed with ImageJ (National Institutes of Health). Where necessary, due to the number of samples, gels were run simultaneously and processed in parallel. The same membranes were used to compare total PDGFRß protein to pPDGFRß.

## Gene expression analysis by qPCR

To analyze RNA expression (**Paper II**), brain tissue was collected as described above. The infarct core and the corresponding contralateral side were homogenized, and the RNA was isolated with the RNeasy mRNA kit (Qiagen). cDNA synthesis was performed using iScript™cDNA Synthesis Kit (BioRad). cDNA was analyzed using real-time PCR SsoAdvanced™ SYBR® Green Supermix from Bio-Rad and run on a Bio-Rad CFX96 real-time quantitative PCR (qPCR) system. Values are presented as mean ± SD of three independent experiments, and within each experiment, triplicate samples were assessed.

## Statistics and data reporting

For the statistical analysis of the data included in this thesis, GraphPad Prism versions 7.0c and 8.0 (GraphPad Software) were used. Data are expressed as mean ± SD and given n-values represent the number of animals used. For two-group comparison Student *t* test was used (**Papers II-IV**) and for multiple group comparison one-way ANOVA (**Paper I**), two-way ANOVA followed by Tukey's

multiple comparisons test (**Paper III**) and multiple t-tests followed by the Holm-Sidak posthoc test (**Paper IV**) were used. Significance was set at $p<0.05$.

Figures were assembled in Adobe Illustrator CS5 version 15.0.0 (**Papers I, III, IV**) or Adobe Photoshop CS5 (**Paper II**).

# Results

A summary of the components of this thesis is presented here. The reader is encouraged to read the full papers for detailed results and figures.

## Pericytes respond early to ischemic stroke

As illustrated earlier, the exact response of pericytes after ischemic stroke remains rather elusive. Therefore, using a permanent stroke model in wild-type mice, we established a detailed timeline of the pericyte and endothelial cell response in relation to the breakdown of the BBB (**Paper I**).

Under physiological conditions, pericytes had a typical pericyte morphology with a round cell body and processes wrapping around blood vessels (**Figure 6a**). We showed that pericytes already responded within 1h to ischemic stroke, whereby they either underwent apoptosis or activation. More specifically, around 50% of all pericytes were positive for the apoptosis marker TUNEL (**Figure 6b**), while the other half were activated, as shown in their expression of NG2 and RGS5 (**Figure 6c** and **Paper I**). Importantly, we did not find any $NG2^{+}$ pericytes that double-labeled with TUNEL, indicating that activated pericytes did not undergo apoptosis (**Figure 6d**). We also detected that pericytes showed signs of detachment from 3h onwards.

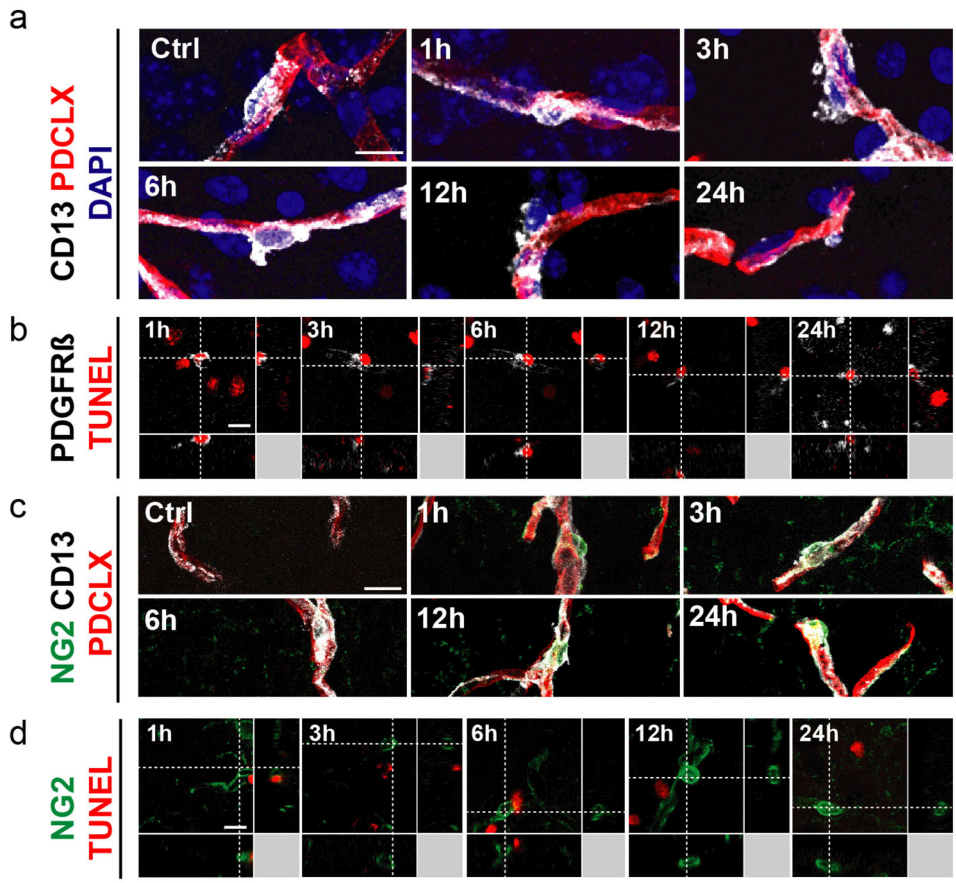


**Figure 6: Pericyte response after stroke includes detachment, activation, and cell death.**
**a.** Representative confocal images of pericytes (CD13, white), vessels (PDCLX, red) and nuclei (DAPI, blue) within the first 24h after stroke showing that after 3h, pericytes start detaching from the vessels. **b.** 3D-representation of TUNEL$^+$ (red) pericytes (PDGFRß, grey) at different timepoints after stroke showing that pericytes die from 1h onwards. **c.** Representative confocal images showing that pericytes (CD13, grey) are positive for the activation marker NG2 (green) from 1h onwards. **d.** No NG2$^+$ (green) pericytes double-label with the cell death marker TUNEL (red). Scale bar 10 µm.

Most importantly, the pericyte response preceded any observable changes in endothelial cells. The first response detected in endothelial cells was a decrease in the protein levels of the TJ proteins zonula occludens (ZO)-1 and occludin, which was observed between 6h and 12h (**Figure 7a, b**). This decrease in TJ proteins was followed by the first detectable endothelial cell death at 12h and a reduction in vessel length at 24h after stroke (**Figure 7c-e**).

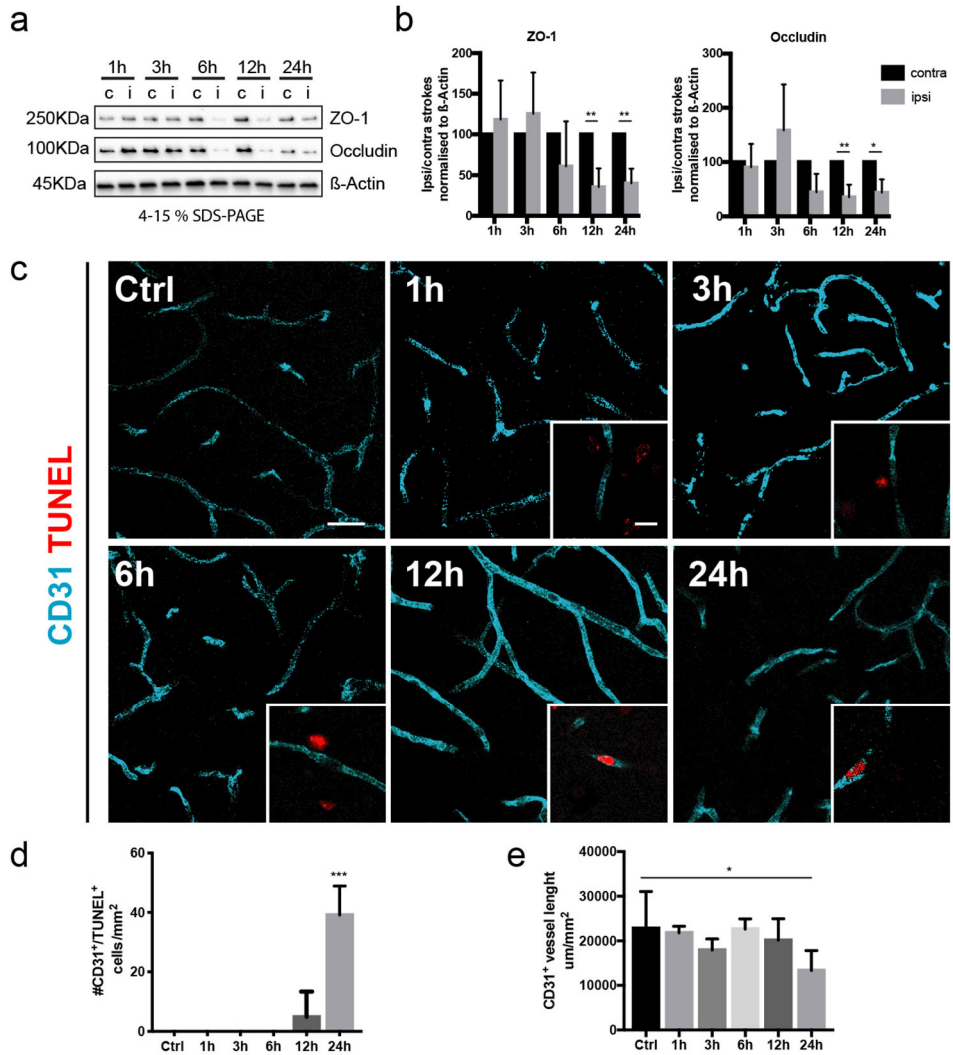


**Figure 7: Endothelial cell response occurs after the pericyte response.**
**a.** WB showing the TJ proteins ZO-1 and occludin of the ipsilateral (i) and contralateral (c) hemisphere at different timepoints after stroke. **b.** Quantification of protein levels of ZO-1 and occludin. ß-Actin was used to normalize the protein content on the gels, and data are expressed as the percentage of ipsilateral/contralateral for each animal. **c.** Confocal images showing the vasculature (CD31, cyan) at different timepoints after stroke. Box in the lower right corner shows that endothelial cells are TUNEL$^+$ (red) at 12h and 24h after stroke. **d.** Quantification of the number of CD31$^+$/TUNEL$^+$ cells. **e.** Quantification showing that the total vessel length is decreased at 24h after stroke. N=4 (WB and vessel length), N=3 (TUNEL) * p<0.05, ** p<0.01, *** p<0.001 (towards all other groups). Multiple t-tests with Holm-Sidak multiple comparison correction for WB. One-way ANOVA with Tukey's multiple comparisons for IHC analysis. Scale bar 20 µm and 10 µm.

Our data showed that the pericyte response occurred hours before the first measurable BBB breakdown, which was assessed both by endogenous leakage of fibrinogen as well as i.v. injected fluorescent-labeled Dextran. Accordingly,

extravascular fibrinogen and Dextran were detected from 12h onwards (**Figure 8,** and **Paper I**). The leakage, however, occurred at the same time as the pronounced decrease in TJ proteins as well as the first detection of endothelial cell death.

Taken together, these data indicate that pericytes are an early responder after stroke reacting to ischemia in different ways. Therefore, pericytes might constitute an important target to prevent BBB breakdown.

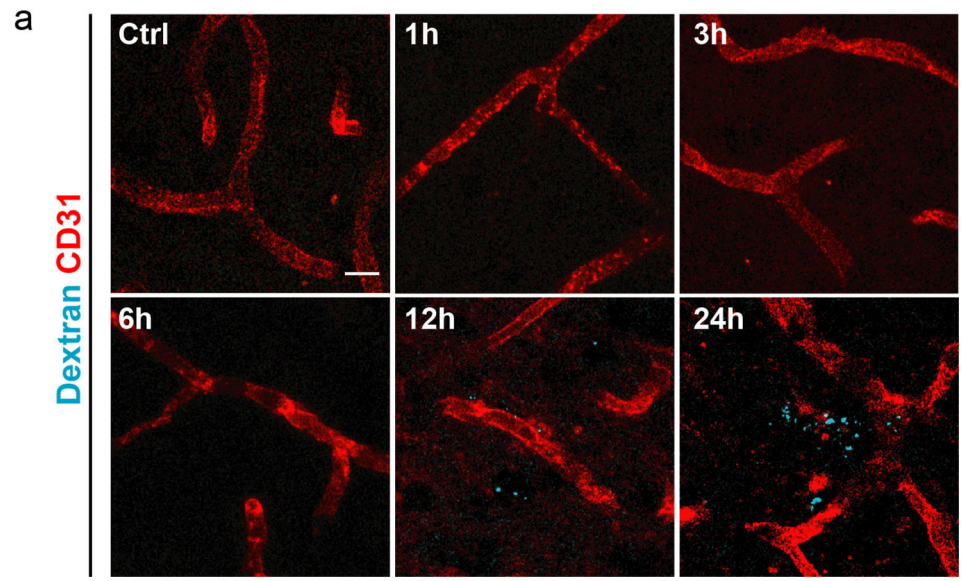


**Figure 8: Vascular leakage occurs at 12h after stroke.**
**a.** Representative confocal images showing extravascular fluorescent-labeled 3 kDa Dextran. Only at 12h and 24h after stroke is there extravascular Dextran (cyan) visible. N=4. Scale bar 10 µm.

# Modulating the pericyte response after stroke

In **Paper I**, we have shown that pericytes are early responders after stroke, and targeting pericyte response might be an interesting approach to prevent or modulate BBB breakdown. We, therefore, next utilized a genetic mouse model, where the brain pericyte-specific *rgs5* gene was replaced with *gfp* [98]. RGS5 is upregulated quickly after stroke (**Paper I**), and it has previously been shown by our group that RGS5 is expressed in detaching pericytes [99]. However, little is known about the role of RGS5 after stroke and whether deletion of *rgs5* modulates the pericyte response after stroke.

## Increased pericyte numbers in RGS5-KO mice leads to neurovascular protection during the acute phase after stroke

We investigated whether loss of RGS5 in brain pericytes modulates the pericyte response in the acute phase after stroke (**Paper II**). RGS5-KO mice had significantly higher numbers of $GFP^+$ pericytes, as well as $PDGFR\beta^+$ pericytes (**Paper II** and **Figure 9a, b**). This increase in pericyte number was further accompanied by an increase in pericyte coverage of capillaries. Additionally, the number of pericytes that expressed the activation marker NG2 was higher in RGS5-KO mice (**Paper II**).

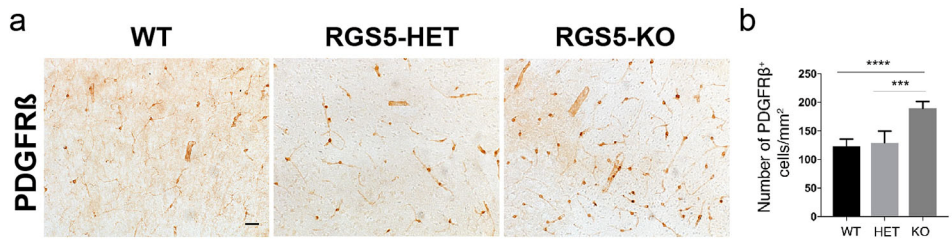


**Figure 9: Loss of RGS5 leads to increased pericyte numbers at 24h after stroke.**
**a.** Representative brightfield images showing $PDGFR\beta^+$ pericytes in the infarct core in WT, RGS5-HET and RGS5-KO mice at 24h after stroke. **b.** Quantification of $PDGFR\beta^+$ pericytes in the infarct core. N=5 (KO, WT), N=4 (HET). *** $p<0.001$, **** $p<0.0001$. One-way ANOVA with Tukey's multiple comparisons. Scale bar 20 µm.

WB for the TJ proteins ZO-1, Claudin-5, and vascular endothelial (VE)-Cadherin showed a decrease in TJ proteins at 24h after stroke in WT mice. This confirmed and expanded previous observations from **Paper I**. Loss of RGS5 in pericytes prevented the decrease of these TJ proteins (**Figure 10a, b**). We further found that RGS5-KO mice had less vascular leakage as indicated by reduced extravasation of i.v. injected Dextran (**Figure 10c**).

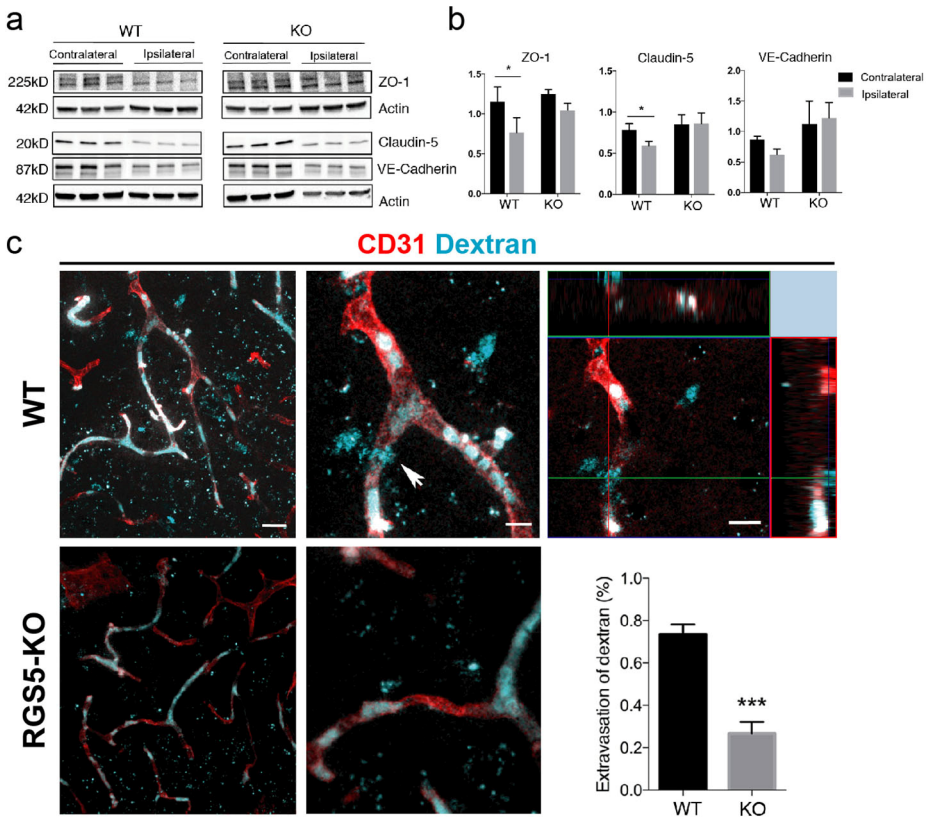


**Figure 10: RGS5-KO mice have preserved TJs and maintained BBB integrity at 24h after stroke.**
**a.** Representative WB of ZO-1, Claudin-5, and VE-Cadherin of the ipsilateral and contralateral hemisphere of WT and KO mice. The contralateral hemisphere of each group served as a control. **b.** Quantification of ZO-1, Claudin-5, and VE-cadherin protein levels, normalized to ß-actin. **c.** Distribution of 10 kDa dextran-tracer (cyan) in the infarct area in WT and KO mice. The middle column shows a higher magnification of the left column. Arrow highlights extravasated dextran. The right column shows a 3D-representation of the perivascular location of dextran. Quantification of extravasation of the 10 kDa dextran of RGS5-KO versus WT mice after stroke. N=3 per group for ZO-1, N=5 per group for Claudin-5 and VE-Cadherin, N=3 for Dextran injections. * $p<0.05$, *** $p<0.01$. Two-way ANOVA with Tukey's multiple comparisons for WB, Student *t* test for extravasation of dextran. Scale bars 20 µm (left) and 10 µm (middle and right).

The reduced BBB breakdown was associated with decreased hypoxia in the infarct core, as determined by a reduced area stained for the hypoxia marker PIMO (**Figure 11a, b)**. Interestingly, cell death after stroke was reduced in RGS5-KO mice, as seen in the reduction of the total number of TUNEL$^+$ cells (**Figure 11a, c)**. Additionally, the percentage of neurons double-labeling with the apoptosis marker TUNEL was reduced in RGS5-KO mice (**Figure 11a, d)**.

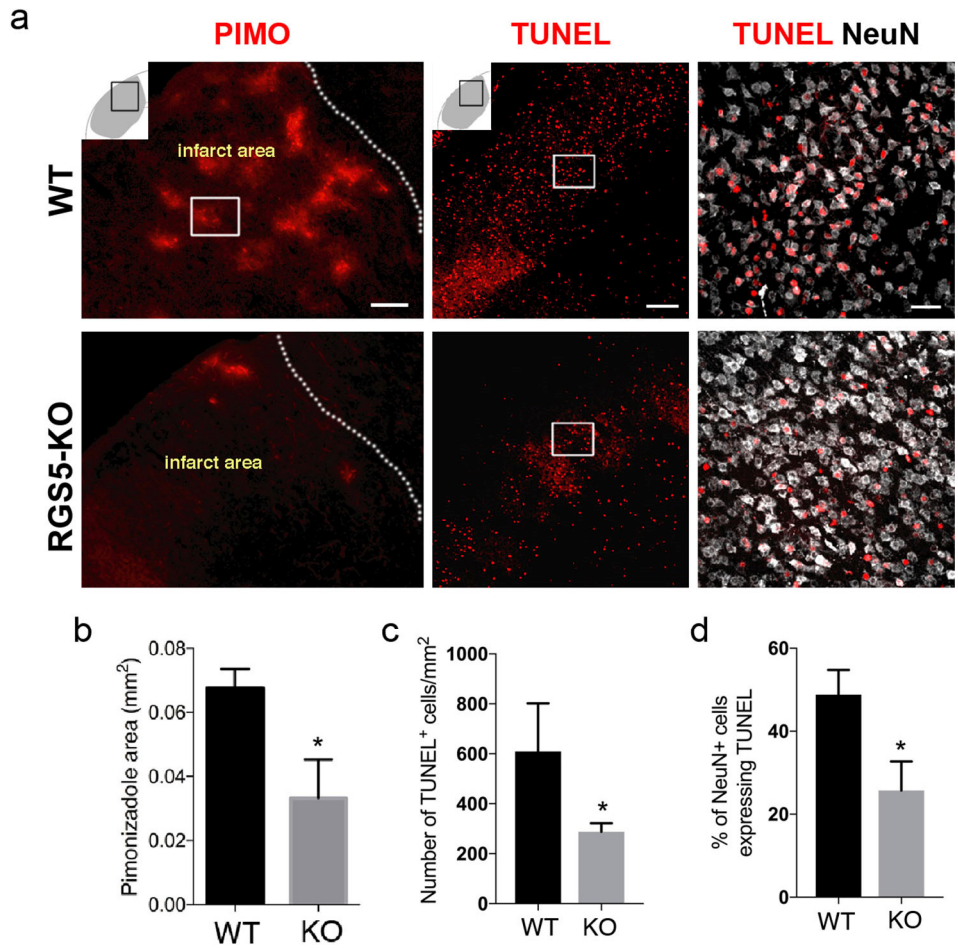


**Figure 11: RGS5 loss in pericytes is associated with reduced hypoxia and increased neuronal survival at 24h after stroke.**

**a.** Representative confocal images of the infarct area of WT and RGS5-KO mice at 24h after stroke. The left column shows the hypoxia marker pimonidazole (PIMO, red) with the infarct area demarcated with a dotted line. The middle column shows the cell death marker TUNEL within the infarct core. The right column shows the apoptosis marker TUNEL (red) with the neuronal marker NeuN (white). **b.** Quantification showing a reduction in $PIMO^+$ area within the infarct area of RGS5-KO mice. **c.** Quantification shows significantly fewer $TUNEL^+$ cells in RGS5-KO versus WT mice. **d**. The percentage of NeuN cells double-labeling with TUNEL is significantly lower in RGS5-KO than WT mice. N=3, *p<0.05, Student *t* test. Scale bar 50 µm (left and middle) and scale bar 20 µm (right).

## Loss of RGS5 results in a shift from a parenchymal to perivascular location of PDGFRß$^+$ cells after stroke

We next investigated whether the increase in pericyte numbers in RGS5-KO mice also had an impact in the chronic phase after stroke. During the chronic phase, pericytes are important key players in various endogenous recovery mechanisms occurring after stroke, including vascular remodeling [28,32]. Further, it has been

shown that PDGFRß$^+$ cells appear in the parenchyma, arguably being pericytes that left the vessel wall and take part in the formation of the fibrotic scar [158,174,175].

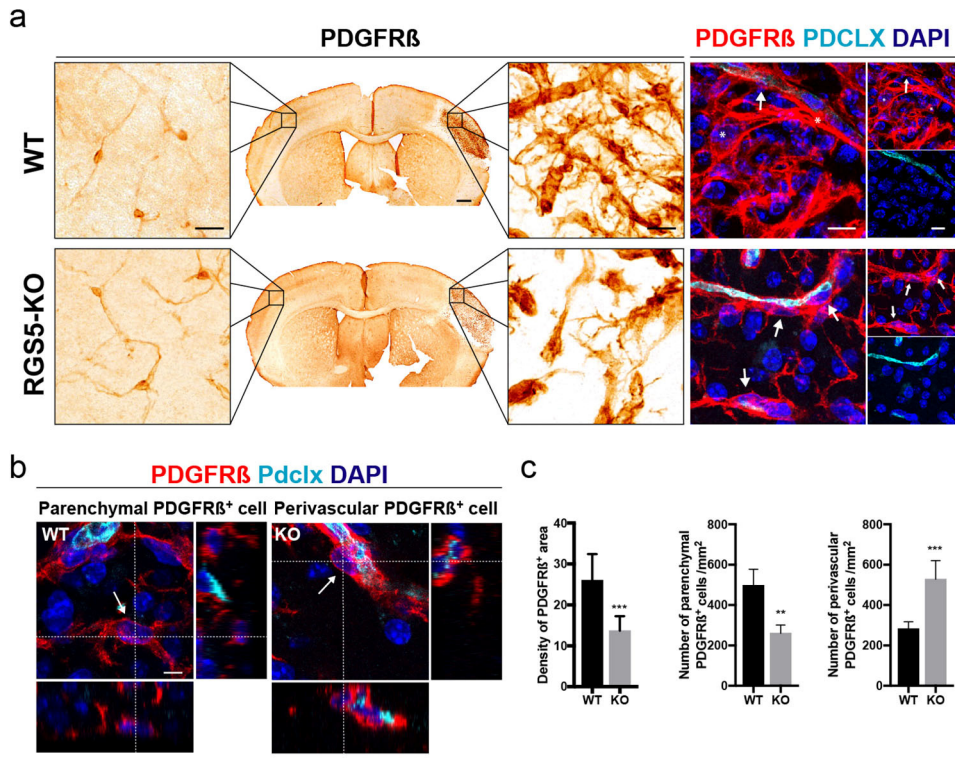


**Figure 12: Loss of RGS5 results in a reduced density of PDGFRß$^+$ cells and increased number of perivascular PDGFRß$^+$ cells at 7 days after stroke.**
**a.** Representative brightfield images of DAB staining of PDGFRß at 7 days after pMCAO of WT and RGS5-KO mice. The left column shows the morphology of PDGFRß$^+$ cells in the contralateral hemisphere, taken as indicated with the boxes in the overview of a brain section shown in the second column. The third column shows higher magnifications of the infarct area. The last column shows confocal images of PDGFRß$^+$ cells (red), the vasculature (PDCLX, cyan), and the nuclear marker DAPI (blue). Arrows indicate perivascular PDGFRß$^+$ cells, and asterisks indicate parenchymal PDGFRß$^+$ cells. **b.** 3D-reconstructions of an example of PDGFRß$^+$ cells (red, with the DAPI$^+$ nucleus in blue) located in the parenchyma (left, as mainly seen in WT mice) and around the vasculature (PDCLX, cyan) (right, as primarily seen in KO mice). **c.** Quantification showing that RGS5-KO mice have a reduced density of PDGFRß$^+$ area, reduced number of parenchymal PDGFRß$^+$ cells, and increased number of perivascular PDGFRß$^+$ cells. N=6, Data are represented as mean±SD. ** p<0 001, *** p<0.0001. Student *t* test. Scale bar 50 µm (overview sections), 10 µm (in a) and 5 µm (in b).

Therefore, we first set out to investigate PDGFRß$^+$ cells in RGS5-KO mice at 7 days after stroke. In contrast to 24h after stroke, the infarct core at 7 days was densely packed with PDGFRß$^+$ cells (**Figure 12a**). Within the infarct core, PDGFRß$^+$ showed two different types of cell morphologies. Perivascular located PDGFRß$^+$ cells had a pericyte-typical round cell body with extensions along the blood vessels and, importantly, were embedded within the basement membrane (**Figure 12a, b** and **Paper IV**). Conversely, parenchymal PDGFRß$^+$ cells had no contact to the

vessel wall and had an amoeboid-like morphology with irregular extensions. We found that RGS5-KO mice had a higher number of perivascular PDGFRß$^+$ cells. However, the area occupied by PDGFRß$^+$ cells in the parenchyma was significantly reduced in RGS5-KO mice, consistent with a decreased number of parenchymal PDGFRß$^+$ cells (**Figure 12a-c**). We also investigated whether loss of RGS5 in pericytes affected PDGFRß-signaling. The ratio of phospho-PDGFRß(Tyr51)/total PDGFRß protein increased in WT mice after stroke, but remained at baseline levels in RGS5 KO mice, indicating that RGS5 mediates changes in PDGFRß-signaling (**Paper III**).

We next studied perivascular PDGFRß$^+$ cells in more detail, and assessed their contribution to vascular remodeling after stroke (**Paper III**).

We found an increased number of perivascular PDGFRß$^+$ cells expressing GFP in RGS5-KO mice (**Figure 13a, b**). Similar to **Paper II**, the number of GFP$^+$ cells was higher in RGS5-KO mice. Loss of RGS5 in pericytes also resulted in increased GFP$^+$ pericyte coverage (**Figure 13a, c**).

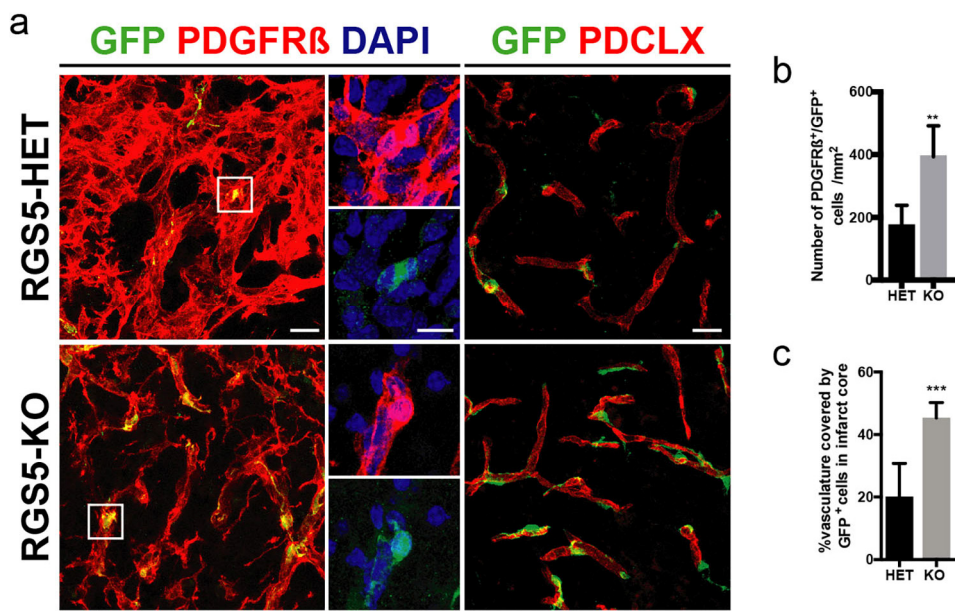


**Figure 13: RGS5-KO mice have higher GFP$^+$ pericyte coverage at 7 days after stroke.**
**a.** Representative confocal pictures of the infarct core of RGS5-HET and RGS5-KO mice. The first column shows an increased number of perivascular PDGFRß$^+$ cells (red) double-labeled with GFP (green). The white boxes indicate where the higher magnification images in the middle column were taken. The right column shows GFP$^+$ pericytes and the vasculature (PDCLX, red). **b.** Quantification of the number of PDGFRß$^+$/GFP$^+$ pericytes in the infarct core. **c.** Quantification of GFP$^+$ pericyte coverage of the vasculature in the infarct core. N=6, Data are represented as mean±SD. **p<0.01, ***p<0.001. Student *t* test. Scale bar 20 µm, and 10 µm in higher magnification.

The shift from a primarily parenchymal to a perivascular location of PDGFRß$^{+}$ cells in RGS5-KO mice had an impact on the vasculature. We observed that RGS5-KO mice had an increased vessel density, as well as preservation of the vessel length in the chronic phase after stroke (**Figure 14a, b**). This was further associated with partial preservation of the BBB, as seen in a reduction of Evans blue extravasation (**Figure 14c**).

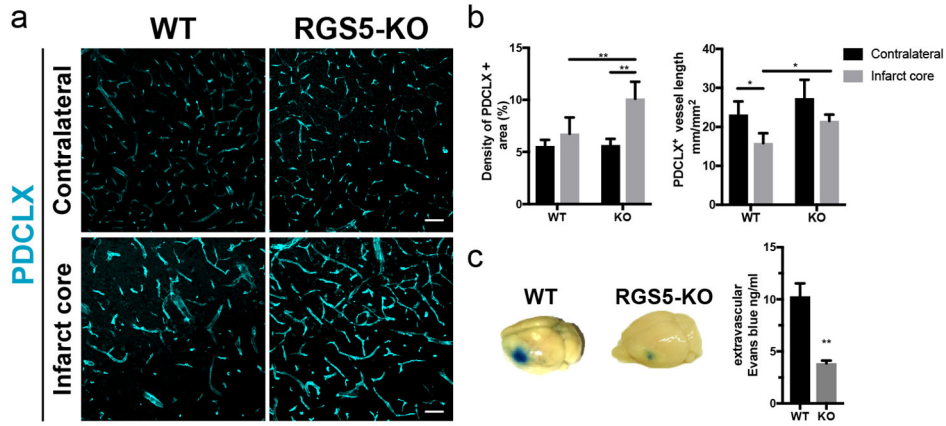


**Figure 14: Loss of RGS5 preserves blood vessels and their integrity at 7 days after stroke.**
**a.** Representative confocal images of PDCLX of WT and KO mice at 7 days after stroke. Upper row shows the contralateral hemisphere and lower row the infarct core. **b.** Quantification of the vascular density (left) and total vessel length (right) of the contralateral hemisphere and the infarct core of WT and RGS5-KO mice. **c**. Representative whole brain pictures of Evans blue leakage at 7 days after stroke of WT and RGS5-KO mice. Quantification of Evans blue leakage. N=6 (Vascular analysis), N=5 (Evans blue). Data are represented as mean±SD. *p<0.05, **p<0.01. Two-way ANOVA with Tukey's multiple comparisons and Student *t* test. Scale bar 40 µm.

## Reduction in parenchymal PDGFRß$^{+}$ cells does not impact on the fibrotic scar formation in the chronic phase after stroke

In the next step, we focused on the parenchymal PDGFRß$^{+}$ cells, as these cells have been described to participate in the fibrotic scar formation [158,175]. First, we confirmed in our model that a fibrotic scar develops within the infarct core, which is surrounded by a glial scar (**Paper IV**).

Using two fibrous ECM markers, Coll-I and FN, that are described to be deposited within the infarct core, we showed that only a small percentage of parenchymal PDGFRß$^{+}$ cells contribute to this deposition. Coll-I was mainly found around the vasculature, while FN was mainly produced by a cell type that did not express PDGFRß (**Figure 15a-d** and **Paper IV**).

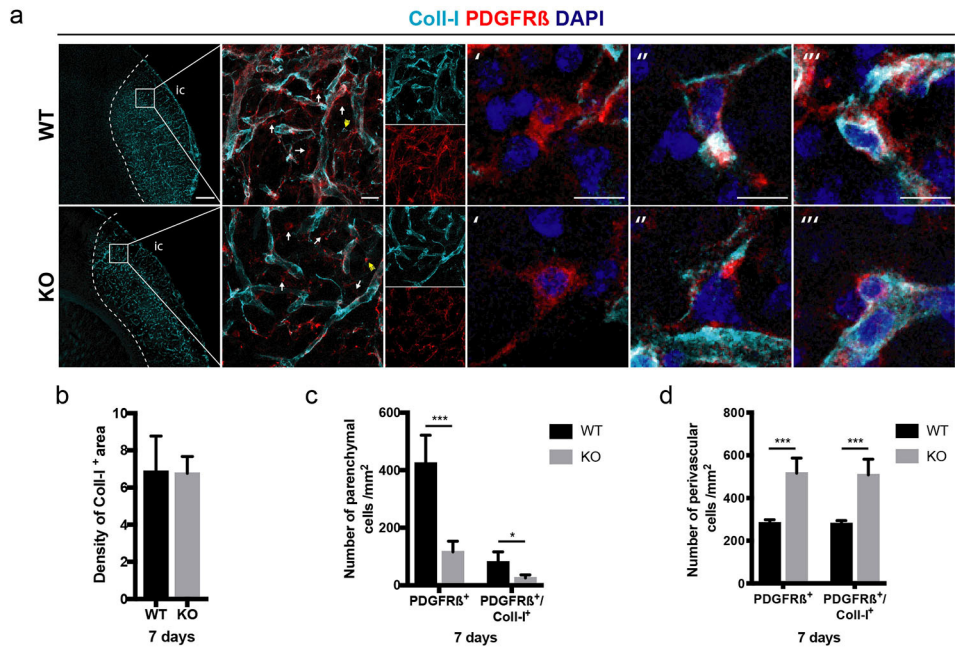


**Figure 15: Parenchymal PDGFRß⁺ cells are not the main contributor to Coll-I deposition after stroke.**
**a.** Confocal images of Coll-I (cyan, left) with PDGFRß (red) and DAPI (blue) at 7 days after stroke. Left column shows an increase in Coll-I within the infarct core after stroke (outlined with dotted lines) in WT and RGS5-KO mice. Boxes indicate that second column images were taken within the infarct core. Second column shows the distribution of Coll-I in relation to PDGFRß staining, with respective single stainings on the right. White arrow indicating that the majority of parenchymal PDGFRß⁺ cells are negative for Coll-I. Yellow arrows indicate the rare presence of parenchymal PDGFRß⁺/ Coll-I⁺ cells. Higher magnification images show a parenchymal PDGFRß⁺ cell that is negative (') and positive ('') for Coll-I, as well as a perivascular PDGFRß⁺ cell that is Coll-I⁺ **b.** Quantification of the density of Coll-I⁺ area. **c.** Quantification of the number of parenchymal PDGFRß⁺ cells that are either positive or negative for Coll-I at 7 days, showing that only few parenchymal PDGFRß⁺ co-label with Coll-I. **d.** Quantification showing an increased number of perivascular PDGFRß⁺ cells in RGS5-KO mice, and that nearly all perivascular PDGFRß⁺ cells double-label with Coll-I. N=5. Data shown as mean±SD. * p<0.05, *** p<0.001. Student *t* test (b) and multiple t-tests with Sidak-Holm post-hoc analysis (c, d). Scale bars 200 µm, 20 µm, 10 µm.

We also investigated the ECM deposition within the vascular basement membrane. The thickening of the vascular basement membrane composed by Coll-IV and laminin was significantly reduced in RGS5-KO mice (**Figure 16a-c** and **Paper IV**).

The infarct core and the fibrotic scar are demarcated by a GFAP$^+$ glial scar (**Figure 17a**). Astrocyte and pericyte crosstalk is important in the regulation of tissue survival [183]; therefore, we finally investigated, whether loss of RGS5 in pericytes affected the formation of the glial scar. We found that at 7 days after stroke, the density of GFAP$^+$ cells in the peri-infarct area was reduced in RGS5-KO mice, which was further accompanied by a reduced number of GFAP$^+$ cells (**Figure 17a-c**). GFAP$^+$ cells in both genotypes had a hypertrophic cell soma. Interestingly, while GFAP$^+$ cells in WT mice had a stellate morphology, GFAP$^+$ cells in RGS5-KO mice had polarized processes towards the infarct core. Additionally, the thickness of the glial scar was significantly reduced in RGS5-KO mice (**Figure 17d**).

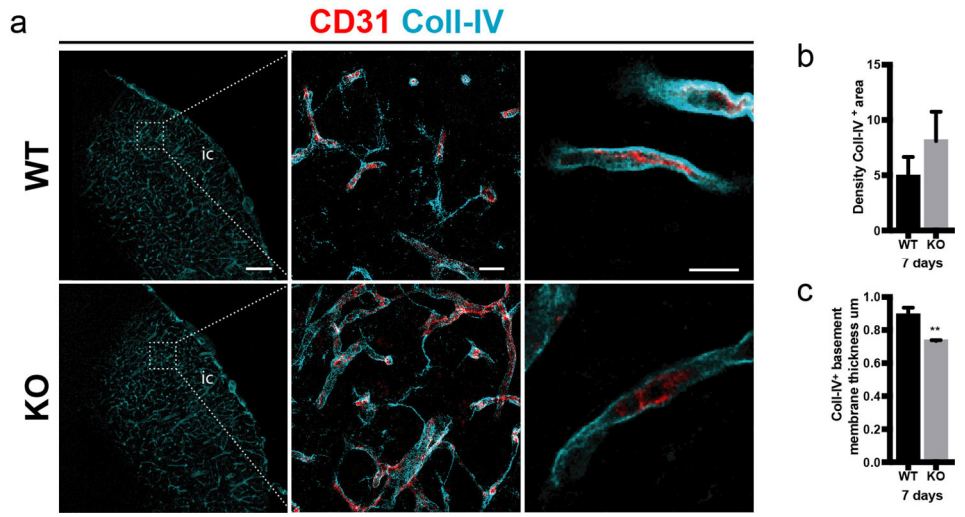


**Figure 16: RGS5-KO mice have a reduced thickness of Coll-IV⁺ vascular basement membrane after stroke.**
**a.** Confocal images of Coll-IV (cyan) and the blood vessel marker CD31 (red) at 7 days after stroke. The left column shows Coll-IV distribution in WT and RGS5-KO mice. The box indicates where the higher magnification picture in the middle column has been taken. The right column shows a high magnification of a single z-stack through a capillary to illustrate the reduced thickness of the vascular basement membrane in RGS5-KO mice. **b.** Quantification of the density of Coll-IV⁺ area at 7 days after stroke. **c.** Quantification of the thickness of the Coll-IV⁺ vascular basement membrane at 7 days. IC: infarct core. N=5. Data shown as mean±SD. ** p<0.01. Student *t* test. Scale bars 200 µm (left column), 20 µm (middle column), 10 µm (right column).

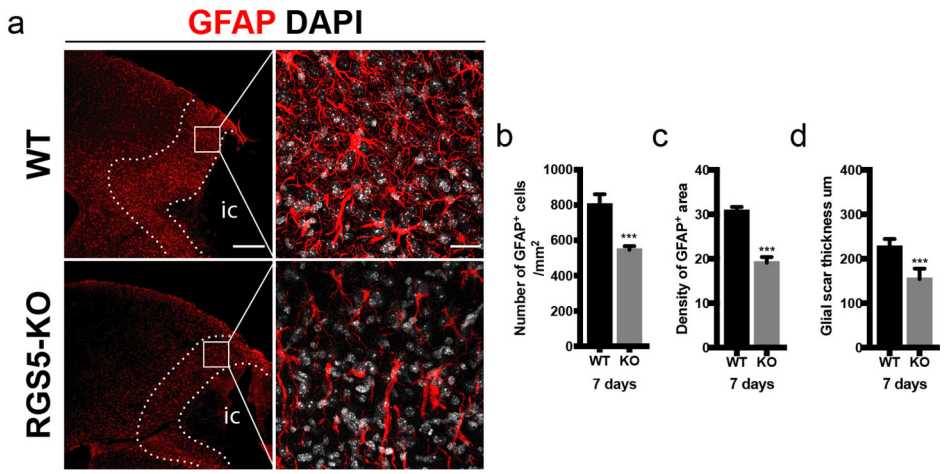


**Figure 17: RGS5-KO show earlier polarization of the glial scar after stroke.**
**a.** Confocal images of GFAP (red) and DAPI (white) at 7 days after stroke. The first column shows an overview of the glial scar. Boxes indicate where the pictures in the second column were taken. **b.** Quantification of GFAP⁺ cell numbers at 7 days in the peri-infarct area showing decreased numbers in RGS5-KO mice. **c.** Quantification of GFAP density in the peri-infarct area at 7 days after stroke. **d.** Quantification of the thickness of the glial scar (as highlighted in overview picture) at 7 days after stroke. IC: infarct core. N=5. Data shown as mean±SD. *** p<0.001. Student *t* test. Scale bars 200 µm (left) and 20 µm (right).

Scar formation is developing over time and can remain for extended periods of time. Therefore, we also investigated RGS5-KO mice at 14 days after pMCAO (**Paper IV**). However, the differences in the redistribution of PDGFRß$^+$ cells were not as pronounced as seen at 7 days. Similar to at 7 days, only a small fraction of parenchymal PDGFRß$^+$ cells double-labeled with either Coll-I or FN. The reduced thickening of the vascular basement membrane, however, was maintained at 14 days. The morphology of GFAP$^+$ astrocytes was similar between the genotypes at 14 days, indicating the establishment of a mature glial scar. While there were no significant differences in the number and density of GFAP$^+$ cells, the glial scar was thicker in RGS5-KO mice, indicating that loss of RGS5 in pericytes results in alteration of the maturation of the glial scar after stroke.

The infarct core increased within the first 24h and, due to tissue constriction, began to shrink at 14 days (**Papers I** and **IV**). Despite the reduced percentage of TUNEL$^+$ cells, reduced leakage, and increased vascular protection, we did not detect any significant differences in stroke size between RGS5-KO and WT mice (**Papers II-IV**), indicating that targeting pericytes by deletion of *rgs5* alone does not result in a reduced stroke size.

# Discussion

In this thesis, we demonstrate that pericytes are early responders to ischemic stroke, and identify pericytes as a potential target to modify the injury progression after stroke.

We showed that the pericyte response preceded endothelial cell death and the breakdown of the BBB. Deletion of *rgs5* in pericytes resulted in an increased number of perivascular pericytes and higher pericyte coverage, leading to reduced BBB breakdown, increased neurovascular protection, and improved vascular stabilization after stroke. Interestingly, reducing the number of parenchymal PDGFRß$^+$ cells did affect the fibrotic scar formation after stroke.

The response of pericytes might differ depending on the stroke model used. Several studies that have previously investigated the pericyte response after ischemic stroke are limited to few timepoints or have used reperfusion models. Reperfusion introduces a secondary injury, the so-called reperfusion-injury, which likely changes the response of vascular cells resulting in a different injury [22,184]. A permanent stroke model was used in all four studies, which results in a focal cortical stroke. The advantage of this model is that it combines a high reproducibility with a low mortality [182,185]. Due to the well-defined lesion, this model allows for the investigation of cellular and neurovascular mechanisms of ischemia without introducing reperfusion. This is also one of the advantages over another commonly used stroke model, the photothrombotic model. The photothrombotic model induces rapid cell death and simultaneous vasogenic and cytotoxic edema, which results in a different cellular response pattern than that seen in human strokes [185,186]. However, due to the relatively small focal lesion, the pMCAO model lacks a clear behavioral readout [182,187].

*Pericytes die or are activated in response to ischemic stroke*

Around 50% of pericytes died within 1h after an ischemic stroke in a pMCAO model (**Paper I**). This finding is in line with previous data demonstrating that in *ex vivo* rat brain slice cultures, the majority of pericytes constricts and dies within 40 minutes after simulated ischemia [156]. Also, Fernández-Klett *et al.* showed a decrease in CD13$^+$ pericytes at 24h after stroke, indicating that pericytes die rapidly after stroke [158]. Our data confirmed the responsiveness to and vulnerability of pericytes in ischemia.

Interestingly, half of the pericytes survived after ischemic stroke (**Paper I**). The surviving pericyte population expressed the marker NG2 and/or RGS5. These markers have been described in the context of activated states of pericytes, such as seen in angiogenesis, vessel stabilization, and vascular remodeling [86,105,108]. It is conceivable that activated pericytes have a role in these processes after stroke.

Pericyte activation, as indicated by the upregulation of RGS5 and/or NG2, is a phenomenon that has been described in a number of neurological diseases. Pericytes are widely activated throughout the brain in response to mouse glioma [104]. In Huntington's disease, pericyte activation is observed before neuronal cell loss and behavioral deficits are detectable [101]. In a mouse model of Parkinson's disease an increased pericyte activation has been described, which interestingly is reversed upon PDGF-BB treatment leading to partial neuronal restoration and behavioral recovery [100].

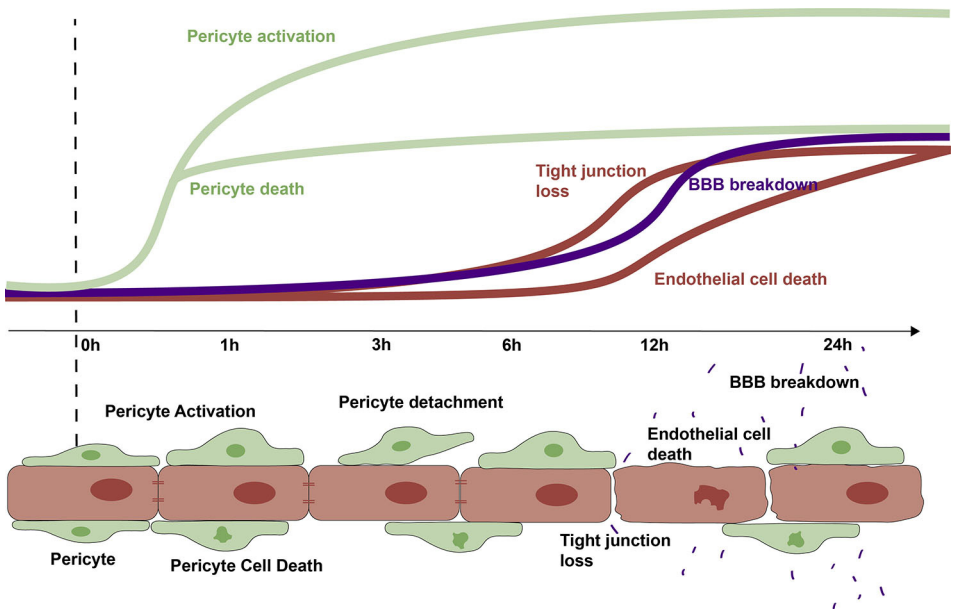


**Figure 18: Timeline of vascular events within the first 24h after stroke.**
Pericyte death and activation are the first observable events after stroke, followed by pericyte detachment. The endothelial cell response occurs later and starts with TJ loss, followed by endothelial cell death and a decrease in vessel length.

*Pericytes respond before endothelial cells to ischemia*

Also following a stroke, pericytes respond very early to the pathological stimulus. In **Paper I,** we showed clearly that the pericytes responded to ischemia before endothelial cells. This is in line with the rapid transcriptional changes seen in pericytes after oxygen and glucose deprivation [188]. Most importantly, the pericyte response preceded endothelial TJ loss and endothelial cell death, as well as vascular

leakage (**Paper I**). This suggests that pericytes are at the beginning of a cascade of pathological events following ischemia. Activated pericytes may act as early sensors of ischemia by mediating inflammatory or other signals to neighboring cells. Similarly, RGS5-expressing pericytes have recently been identified as early sensors of systemic inflammation where they relate inflammatory signals to neurons [189].

The time window between the rapid pericyte response to the insult and the delayed endothelial cell death and BBB leakage opens the possibility to target pericytes with the intent to prevent further damage associated with BBB breakdown. Reducing pericyte cell death or detachment might constitute a therapeutic strategy to prevent BBB breakdown after stroke (**Figure 18**).

*RGS5 as a target to modulate the pericyte response*

In this thesis, we showed that RGS5 in pericytes is a potential target to modulate the pericyte response. The rationale for using RGS5 was several fold: It was based on our previous observation that pericytes that detach from the vessel wall express RGS5 [99]. Furthermore, since RGS5 is expressed in activated pericytes under pathological conditions, we hypothesized that the deletion of *rgs5* might lead to maturation of pericytes. In RGS5-KO mice, the number and location of pericytes were not altered under physiological conditions. After stroke however, loss of RGS5 in pericytes resulted in increased numbers of perivascular pericytes and reduced numbers of parenchymal PDGFRß$^+$ cells (**Papers II-IV**). This provided us with a model to study the consequences of changes in pericyte numbers, detachment, and redistribution of pericytes after stroke.

Several mechanisms could underlie the increase in pericyte numbers observed in RGS5-KO mice. First, RGS5 has been suggested to be involved in pericyte detachment at 7 days after stroke, but RGS5 seems to be important already in the detachment observed within hours after stroke [99]. Our data showed that detachment might require RGS5, indicated by the reduced number of parenchymal PDGFRß$^+$ cells in RGS5-KO (**Paper III**). Loss of RGS5 may interfere with the process of detachment and keep pericytes attached to the vessel wall, suggesting that RGS5 protein expression is needed for pericyte detachment. Second, the loss of RGS5 might increase the survival of pericytes. This hypothesis is supported by our finding that none of the GFP$^+$ pericytes in RGS5-KO mice underwent apoptosis (**Paper II**). Third, higher numbers of pericytes in the infarct core of RGS5-KO mice may also be due to increased proliferation. This is in line with an *in vitro* study showing that RGS5 inhibits proliferation of human coronary artery SMCs [190]. Fourth, the increase in pericyte numbers could be due to enhanced migration of pericytes towards the injury site. In support of this, it has been shown that downregulation of RGS5 results in enhanced migration *in vitro* [191]. Lastly, enhanced pericyte recruitment to newly formed vessels might contribute to the increased number of perivascular pericytes. This is supported by the high expression of PDGFRß around blood vessels, which is required for pericyte recruitment [75].

*Targeting pericytes protects the BBB integrity after stroke*

TJ loss, the first observable response in endothelial cells, was an early indication of BBB dysfunction (**Paper I**). The timepoint of TJ degradation in relation to BBB breakdown after stroke differs between different studies. Knowland *et al*. showed in a transient MCAO model that TJs are impaired only after 2 days, despite BBB dysfunction occurring at 6h after stroke [192]. Other studies have shown a decrease as early as 3h after stroke [21,193]. This indicates that different timepoints of reperfusion might have a substantial effect on TJ loss after stroke.

The loss of TJ may be caused by a number of factors. It could be due to a direct effect of hypoxia or caused by degradation via hypoxia-activated proteases [194-197]. Additionally, pericytes are important in the maintenance of TJs, and the loss of pericytes itself likely contributes to the decrease of TJs [198]. As the loss of pericytes preceded the decrease in TJ proteins, increasing the pericyte number prevented this decrease in TJs in RGS5-KO mice (**Papers I** and **II**). These findings support that pericytes are crucial to maintain TJs. Consistent with our findings, pericyte numbers in the spinal cord correlate with the expression of ZO-1 and occludin [199]. Similarly, postnatally induced PDGFRß-KO mice, which show a reduction in pericyte numbers, have a decreased expression of TJ proteins, as well as deformed TJ after stroke [164,165]. Our data also added to previous findings showing that prevention of pericyte loss is associated with reduced TJ degradation after lysophosphatidylcholine-induced neurodegeneration [200].

Increasing perivascular pericyte numbers after stroke reduced BBB breakdown (**Papers II** and **III**), likely through preventing TJ degradation [201-204] . BBB breakdown after stroke is biphasic, with an initial breakdown early in the acute phase, followed by a second opening during angiogenesis in the chronic phase after ischemic stroke [19,37,192]. We found that loss of RGS5 in pericytes had an impact on the BBB leakage during both the acute and chronic phase after stroke. Therefore, increasing pericyte numbers and coverage through *rgs5* deletion could be an approach to prevent BBB breakdown (**Figure 19**). This is supported by a study showing that reduced BBB breakdown is associated with increased pericyte numbers at 21 days after stroke [204]. Additionally, several studies have shown that loss of pericytes is associated with BBB breakdown [164,165,205]. This is especially interesting in the light of contradictory studies that have shown that pericytes might not only be protective for the BBB but might themselves negatively influence the BBB integrity. In this regard, pericytes have been discussed to contribute to the BBB breakdown by expressing Nox4 and VEGF, and by their gelatinase and MMP-9 activity [168,206,207]. However, the data provided in this thesis supports that a decreased number of perivascular pericytes contributes to the BBB breakdown after stroke (**Papers II** and **III**).

RGS5-KO mice showed reduced neuronal cell death, likely as a result of the reduced BBB breakdown (**Paper II**). BBB breakdown results in the accumulation of blood-

derived toxins in the brain parenchyma which further exacerbates the brain damage after stroke. Neuronal protection might therefore be the result of decreased extravasation of toxic molecules, or the reduced level of hypoxia observed in RGS5-KO mice (**Paper II**). Consistent with our findings, BBB breakdown has been shown to be linked with increasing hypoxia levels, which in turn are closely associated with neuronal damage [208,209]. Additionally, human brain pericytes secrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF) *in vitro* and thus, might contribute to neuroprotection and neuroregeneration [123,136,137,210].

However, the partial BBB preservation and the reduction in neuronal cell death did not translate into changes in stroke size within the first 2 weeks in the stroke model used (**Papers II-IV**). This could indicate that targeting the pericyte response by deleting *rgs5* alone is not sufficient to prevent or recover from a stroke. Whether loss of RGS5 could reduce the stroke size in more severe stroke models remains to be investigated.

Despite this, our findings highlight the potential of preventing BBB breakdown by targeting pericytes. Pericyte loss during aging and in neurodegenerative diseases has been shown to result in enhanced BBB breakdown that aggravates neurological dysfunction [122,123,149,211]. Therefore, pericytes are also an interesting target for a variety of other neurological disorders.

*Targeting pericytes to enhance vascular stabilization*

The decrease in endothelial TJ not only coincided with the BBB breakdown but also with the onset of endothelial cell death after stroke (**Paper I**). The subsequently observed reduction in vessel length could be a direct result of the endothelial cell death [212]. Our data suggests that pericyte loss after stroke can aggravate endothelial cell death and negatively impact on the vasculature (**Figure 19**). This might be due to a protective effect of pericytes on endothelial cells, as pericytes promote endothelial survival as well as reduce their responsiveness to hypoxia [213,214]. Accordingly, pericytes promote endothelial survival through several signaling pathways, including PDGF, Sphingosine-1-phosphate, VEGF-A, and Ang1/Tie2 [215-219].

Increasing the pericyte number and coverage in RGS5-KO mice resulted in increased vessel density and preserved vessel length after stroke (**Papers II** and **III**). There are be several mechanisms that could underlie this increased vessel density seen in the chronic phase of RGS5-KO mice. It could either be a consequence of the preserved vasculature early after stroke due to enhanced survival of endothelial cells or due to increased vascular remodeling. In support of the latter, we found a decreased thickening of the vascular basement membrane (**Paper IV**) which could contribute to vascular remodeling, as Coll-IV in particular has been described to inhibit angiogenesis [220,221].

In a study of hereditary hemorrhagic telangiectasia, which is characterized by vascular malformations leading to recurrent nosebleeds, treatment with the drug thalidomide resulted in vessel maturation [222]. Most interestingly, this enhanced vessel maturation was associated with increased mural cell coverage [222]. The same drug also increased mural cell coverage in a mouse model of brain arteriovenous malformation and thereby reduced hemorrhage [223]. This indicates that increased pericyte coverage is a useful target in vessel stabilization. In line with this, transplantation of human pluripotent stem cell-derived pericytes improved vascular and muscle regeneration in a mouse model of hind limb ischemia [224].

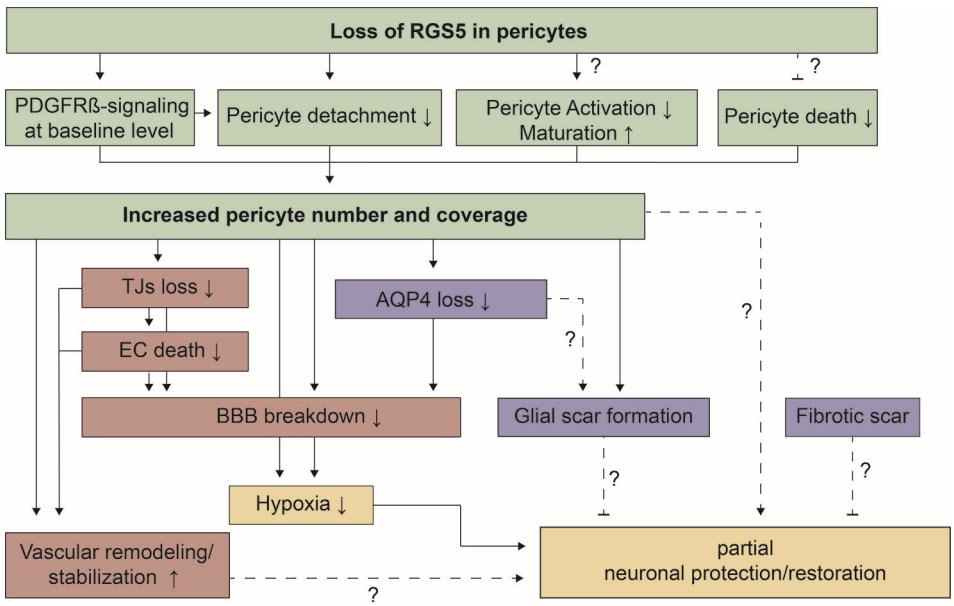


**Figure 19: Possible mechanisms involved in the modulated pericyte response by *rgs5* deletion after stroke.** Loss of RGS5 in pericytes results in increased pericyte coverage, possibly through several mechanisms, including maintaining PDGFRß-signaling at baseline levels and reduced pericyte detachment. The increased pericyte coverage prevents TJ loss and endothelial cell death, which results in reduced BBB breakdown. Further, increased pericyte coverage prevented AQP4 loss and increass vascular remodeling and stabilization. Reduced BBB breakdown likely contributes to reduced hypoxia and partial protection of neurons. Increased pericyte coverage does not affect the fibrotic scar. Lines indicate a connection based on data provided in this thesis and dotted lines a connection that is based on speculations inspired by results and literature.

There could be several underlying mechanisms explaining the increased vascular stabilization in RGS5-KO mice. Accordingly, overexpression of RGS5 has been shown to inhibit sonic hedgehog (Shh)-mediated signaling, which reduces the recruitment of pericytes as well as vessel maturation, opening the possibility of RGS5 to act on PDGFRß-signaling through Shh [225,226]. Further, maintaining PDGFRß-signaling at baseline level could indicate a mature and normalized phenotype of pericytes after stroke, hence reducing the number of PDGFRß$^{+}$ cells in the parenchyma. Phosphorylation of PDGFRß at Tyr571 leads to

phosphoinositide 3-kinase (PI3K) binding, which mediates actin reorganization, migration, and differentiation [79,227-229]. RGS5 may directly or indirectly interact with the receptor or the corresponding downstream pathways. However, further studies are needed to dissect the impact of the loss of RGS5 on the downstream signaling pathways leading to pericyte detachment.

Vascular remodeling and maturation of newly formed vessels is also of interest in other pathologies, such as tumors. Loss of RGS5 in pericytes has been shown to result in vascular normalization through pericyte maturation in a tumor model [107]. In particular, RGS5 is upregulated in tumor vessels in a HIF-dependent manner, while antitumor therapy downregulated RGS5 and loss of RGS5 resulted in pericyte maturation, leading to vascular normalization and reduced tumor hypoxia and vessel leakiness [105,107]. Despite differences in vascular remodeling in stroke and tumors, this highlights that pericytes are an important cell population to target vascular remodeling and stabilization under hypoxic conditions.

Finally, injury and dysfunction of endothelial cells are implicated in the pathogeneses of diverse vascular diseases [230]. Preventing or reversing these injuries through the deletion of *rgs5* in pericytes might offer a novel therapeutic target in a variety of diseases. In support of this, we have previously shown that PDGF-BB treatment had neurorestorative effects in a mouse model of Parkinson's disease, which, most importantly, was associated with a normalized pericyte response likely allowing for vascular stabilization [100]. Interestingly in this regard is another study, showing that PDGF-BB treatment *in vitro* results in downregulation of RGS5 [191].

*Contribution of PDGFRß$^+$ cells to scar formation*

The detachment of pericytes occurs at different stages after stroke [158-160]. Our data is in line with previous work showing that pericytes detach within the first hours after stroke [159,160]. Pericyte detachment not only plays a role in BBB breakdown and angiogenesis; previous studies have suggested that detached pericytes migrate into the parenchyma, change their phenotype and might contribute to the inflammatory response and fibrotic scar formation in the chronic phase after ischemic stroke [99,158].

Surprisingly, despite a 50% reduction of parenchymal PDGFRß$^+$ cells, we did not detect any changes in the deposition of the ECM proteins Coll-I and FN (**Paper IV**). Only a small fraction of the parenchymal PDGFRß$^+$ cells colocalized with Coll-I and FN, suggesting that parenchymal PDGFRß$^+$ cells are not the main contributor to fibrous ECM deposition after stroke. Other studies have reported higher co-labeling of PDGFRß$^+$ cells with at least FN, although the authors did either not distinguish their specific location or quantify cell numbers [158,175]. Additionally, the usage of either reperfusion or photothrombotic stroke models makes it difficult to compare results directly [158,175]. To date, Göritz *et al.* provided the only lineage-tracing study performed in the CNS, suggesting a pericyte origin of scar-forming cells in the spinal cord using a GLAST-promoter [178]. Most interestingly, reducing

pericyte-derived fibrotic scar tissue promotes functional recovery after spinal cord injury [231]. This difference to our study might be explained by targeting different subpopulation of pericytes, as well as different developmental origins of cortical and spinal cord pericytes [59-61,178]. Differences in the developmental origins of pericytes might also partially explain the controversy of the pericyte contribution to fibrosis in different types of tissues. As an example, two studies using different lineage tracing approaches came to contradicting results regarding the origin of scar-forming myofibroblasts in kidney fibrosis [177,232]. This controversy is also reflected within the CNS, where in addition to pericytes, also perivascular, meningeal, or circulating fibroblasts have been proposed as the source of scar forming-cells [233-236]. We cannot conclude whether all parenchymal PDGFRß$^{+}$ cells originate from pericytes after stroke, as only lineage tracing can fully address this question. However, our study showed that targeting a brain pericyte-specific gene results in a redistribution of the location of PDGFRß$^{+}$ cells, supporting their pericyte-origin.

It is worth noting that our data indicated that loss of RGS5 in pericytes in itself did not directly affect Coll-I and FN production. This is interesting considering that RGS5 has been implicated in modulating ECM production during fibrosis in the liver and heart [108,237,238]. This suggests that the described role of RGS5 in ECM production might not be directly applicable to brain pericytes after stroke. A possible explanation for these differences could be that the ECM within the brain has a different composition to that in other tissues [41-43]. The fibrotic scar has been suggested to impede the anatomical plasticity within the CNS and therefore, could impact negatively on functional recovery after stroke. Therefore, the finding that targeting pericyte response by *rgs5* deletion does not impact on the fibrotic scar after stroke might be one of the contributing factors explaining why RGS5-KO mice did not exhibit a reduced stroke size.

Interestingly, we observed changes in the glial scar formation. The redistribution of PDGFRß$^{+}$ cells in RGS5-KO mice resulted in an earlier polarization of the glial scar, suggesting a potential cross-talk between pericytes and astrocytes after stroke. In line with this, studies in either pericyte-deficient or aquaporin-4 deficient mice have shown that pericytes influence the polarization of astrocytes under physiological conditions [50,180]. Further, the cell-cell communication between astrocytes and pericytes is necessary for BBB maintenance [183,239]. Their cross-talk is further highlighted by studies showing that GFAP-KO mice compensate for the lack of astrocytic end-feet by increased pericyte coverage and proliferation [183]. Additionally, PDGFRß-KO mice develop a disrupted glial scar after stroke [162]. However, changes in the glial scar could be secondary, as the formation of the glial scar is closely coordinated with the inflammatory response and the BBB breakdown [44,240-242]. Therefore, the reduced BBB breakdown in RGS5-KO mice (**Paper II** and **III**) could explain the changes in the glial scar maturation.

A glial scar forms in several CNS disorders, including stroke, chronic neuroinflammation, traumatic brain injury, and brain tumors [44]. As glial scar

formation has been described to have a dual role, on one hand limiting the spread of inflammation and on the other hand inhibiting functional recovery, modulating the glial scar might improve functional recovery [29,241,243].

# Conclusion and future perspectives

We provide novel evidence for brain pericytes as an essential target for protection and repair of the cerebral vasculature, with the potential to positively impact on stroke outcome. We identified a detailed timeline of the response of pericytes to stroke and addressed key functions of pericytes during the acute and chronic phase of stroke by modulating this pericyte response. Therefore, this thesis emphasizes pericytes as key players in stroke pathology and as a novel target cell type at the top of the pathological cascade after the ischemic insult. This thesis also significantly contributes to a better understanding of how pericytes can be targeted in the future to prevent BBB breakdown and enhance vascular remodeling, possibly not only in stroke but also other neurological disorders.

One of our key findings identifies brain pericytes as an early responder to ischemic stroke. These results imply that pericytes act as an early sensor to ischemia, likely due to their strategic location at the interface between blood and brain. In this regard, it would be interesting to investigate the early transcriptional changes of pericytes in response to stroke. This could shed light on the molecular mechanism leading to pericyte death or survival. Additionally, dissecting changes in the pericyte secretome and how this impacts on stroke pathology would be of great interest. Together with the transcriptional changes, this could pinpoint which signals pericytes relay to surrounding cells and how this might influence the pathology after stroke.

Another key finding identifies RGS5 as an important modulator of neurovascular protection and vascular remodeling after stroke. We establish an essential role of RGS5 in the breakdown of the BBB, and our studies suggest that RGS5 is an important modulator of pericyte detachment. Accordingly, loss of RGS5 is involved in the shift from a parenchymal phenotype to a perivascular phenotype resulting in a preserved vasculature after stroke. For the future, it would be interesting to investigate whether the loss of RGS5 prevents pericyte death and/or regulates the secretome of pericytes to understand how the loss of RGS5 in pericytes affects stroke development and outcome beyond the morphological changes determined in this thesis. Especially clinically relevant would be to verify our results by blocking RGS5 in wild-type mice, as well as to investigate in which way reperfusion changes the pericyte response after stroke.

Additionally, our data sheds light on the current question of whether pericytes contribute to the fibrotic scar formation after stroke. We show that parenchymal PDGFRß$^{+}$ cells are not the main contributor to the fibrotic ECM deposition. Therefore, targeting these cells via RGS5 might not impact on fibrotic scar formation after stroke. Performing lineage-tracing experiments will help to clarify the role of pericytes in the fibrotic scar formation. Also, assessing additional ECM proteins might further add information regarding the contribution of PDGFRß$^{+}$ cells to the scar formation. It would be interesting to investigate the underlying mechanism of how pericytes influence the formation of the glial scar. This knowledge could help to modulate the glial scar and hence improve functional recovery.

Our studies add to the increasing evidence that pericytes are an important cell type in neurological diseases. Targeting pericyte location and numbers, for example via deletion of *rgs5*, might be an opportunity for therapeutic interventions in order to prevent BBB breakdown and to promote vascular stability also in other acute neurological disorders. Loss of RGS5 may also impact on pericyte detachment in more chronic diseases such as Parkinson's and Huntington's disease that have shown pericyte activation and BBB leakage.

Overall, this thesis identifies pericytes as a key player in stroke. As pericytes are at the top of the pathological cascade after the ischemic insult, targeting pericytes could be of clinical relevance for the development of future therapies after ischemic stroke.

# Acknowledgments

I would like to take the opportunity to express my gratitude to everybody who had an impact on the years of my PhD studies and, hence, made this work possible. Without the support and encouragement, you would not hold this book in your hands today.

First and foremost, I would like to thank **my main supervisor, Gesine Paul-Visse**, for taking me on as a PhD student and introducing me to the exciting field of pericytes. Thank you for letting me work on many different projects and for your guidance throughout the years. Thanks for teaching me the importance of a good story for every piece of writing and presentation, and for staying calm and optimistic in times of crisis. A special thank goes to **my co-supervisor Maria Björkqvist**, for all your kind words of encouragement over the last 4.5 years.

I am particularly grateful to have had the chance to worked together with the wonderful **TNY** group. I would like to thank all its past and present members; **Ilknur** for teaching me all the relevant lab work and guidance, **Marco** for making the office a happy place**, Abderahim** for your valuable feedback, **Robert** for all the emergency help and random conversations, **Osama** for your kind words of encouragement, **Thomas** for your support and advice on how to manage to get to this point, and **Andreas** for the fun times and proofreading of so many pieces of work! Thank you all not only for your guidance and contributions to the scientific work but also for your impact on a personal level. And a big thanks for the help during the writing process of this thesis!

Moving to a new country without knowing anybody was a big step, but I was fortunate to join A10, where I felt welcome from the very first moment. Over the years, A10 has seen many people, all of which have contributed in one way or another to my PhD experience. To **all past and present members of A10**, thank you for valuable tips and practical help, and especially for the hours in the sauna, countless glasses of whiskey and wine on Fridays and all the fun memories along the way! **Carla, Margarita, and Patrick**, words cannot describe how much you shaped my life during those early years, and A10 was never the same again after you left! A special thank goes to **Andy** for reading through this entire book! **Caroline and Andreas**, thanks for the sunshine and ego you brought into my life; the last year would have been a very rainy one without the two of you! And thank

you Caroline for your effort in taking a nice picture of me! Thanks also to **all BMC friends and colleagues** outside A10.

**Alicja**, thank you for everything! You were such a great support, starting from teaching me how to staining, to so perfectly cut all those brains, while being the angel of A10! **Susanne, Marianne, AnnaKarin, Ansa, and Katarina**, thank you very much for all the technical and organizational help during my PhD, it would have been much harder without all your support!

Tack så mycket till **alla sjuksköterskor från Stroke register**, det har varit roligt att arbeta med er och jag kommer att sakna telefonsamtalen med er alla.

I'm very glad I could work alongside with **Selvi, Shelby, and the rest of MDR** to raise the students' voices and shape our PhD education. It was so much fun to organize all those events with you, and to deal with one or two crises along the way!

**Daniela, Jana, and Paula**, I'm so happy you joined SymBioSE in Greece and convinced me of our potential. **Svetlana, Marta, and Xico**, thanks for joining our team, and making SymBioSE happening in Sweden. It was such an incredible experience, and I still can't believe we pulled it off so perfectly! I'm also extremely thankful for all the relaxing knitting nights that followed afterwards together with **Gjenni.**

Thank you to all the people I meet over the years outside work, that kept reminding me that there is a life outside the lab! A huge thank goes to all the **Swedish friends**, for integrating me, being patient with my Swedish skills, and for making sure I experience all the Swedish traditions properly, even if I still don't like kräftor.

Ohne die Unterstützung meiner Familie wäre dieser PhD nie möglich gewesen. **Mama und Papa**, vielen Dank, dass ihr mich ermutigt habt die Welt zu erkunden, und mir die Flügel dazu gegeben habt. Ich kann euch nicht genug für eure Unterstützung danken, und dass ich immer weiss, dass ich ein sicheres Base-Camp in Wiler habe. **David**li, danke, dass du mein grösserer Fan bist!

**Sten**, without you, this PhD would have been so much harder. I can't thank you enough for your constant encouragement and for believing in me. Thank you so much for reminding me to enjoy life, and for exploring the world near and far, both on land and water, and climbing up mountains no matter how high together with me. And for making sure I eat more than just yogurt along the way!

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*I think*
*I'm quite ready*
*for another*
*adventure.*

- Bilbo Baggins

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

# About the Author

Michaela Roth studied Biology at the University in Basel (Switzerland) and received a Masters's degree in Molecular Biology. She started her PhD in Neuroscience at Lund University within the Translational Neurology Group in 2015. Her work centered on elucidating the role of pericytes and the impact of RGS5 loss on pericyte response after stroke.

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LUND UNIVERSITY

FACULTY OF MEDICINE

Translational Neurology
Department of Clinical Science Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:99
ISBN 978-91-7619-828-5
ISSN 1652-8220